experimental control, no general satisfactory definition of 'limiting enzyme' can be made. In the case of an enzyme catalysing the irreversible decomposition of a single substrate, it will clearly limit the overall rate of fermentation when it is saturated with respect to its substrate. When the yeast we have studied ferments excess glucose at 30° carboxylase very nearly meets this requirement: according to the simple interpretation of the results we have suggested, this enzyme system is about 80% saturated by 12 μmoles pyruvate/g. yeast. It should be noted that this does not imply that raising the activity of the carboxylase system would necessarily result in an increased fermentation rate: what is implied, however, is that no substantial rate increase is possible unless the activity of intracellular carboxylase is increased through synthesis of specific protein, or of coenzyme, or even possibly by alteration of physico-chemical properties of the enzyme milieu, such as pH.

SUMMARY

1. The intracellular pyruvate level of yeast has been measured as the difference between the total pyruvate extracted from a suspension by trichloroacetic acid and that excreted into the medium.

2. The relation between fermentation rate and intracellular pyruvate resembles that between the velocity of an enzyme reaction and substrate concentration.

3. The velocity of the carboxylase system in metabolizing yeast suspensions may therefore be deduced from pyruvate measurement when direct measurement by determination of carbon dioxide output is not feasible.

4. A possible interpretation of the results as a direct determination of the kinetics of the carboxylase system *in vivo* is given.

5. The limitation of fermentation rate, in the yeast studied, by carboxylase is discussed.

The authors wish to thank the Directors of the Distillers Co. Ltd. for permission to publish this paper.

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Studies on Yeast Metabolism

4. THE EFFECT OF THIAMINE ON YEAST FERMENTATION

By W. E. TREVELYAN and J. S. HARRISON
Research and Development Department, Distillers Co. Ltd., Epsom, Surrey

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Since the discovery of the acceleration of alcoholic fermentation by thiamine (Schultz, Atkin & Frey, 1937*a*) and the development by these authors (see their review in Anderson, 1946) of their fermentation test, several attempts have been made to establish the nature of the effect. The problem is complicated by the fact that not all yeasts respond to thiamine in the same way, varying periods of incubation being required (e.g. Laser, 1941).

According to Sperber & Renval (1941) thiamine is taken up by yeast in two phases; a rapid absorption is followed by a slower conversion of absorbed thiamine into cocarboxylase. Westenbrink, Steyn-Parvé & Veldman (1947) also studied the conversion of thiamine into cocarboxylase by living yeast, but found that although 200–300 μg. of cocarboxylase could accumulate per gram of yeast, no increase in the enzyme carboxylase could be demonstrated.

Went (1949) on similar grounds, and also because the pyrimidine part of the thiamine molecule, which had been shown to accelerate fermentation (Schultz, Atkin & Frey, 1937*b*), apparently did not form cocarboxylase, suggested that the effect of thiamine on the fermentation rate did not involve the carboxylase system. An indirect effect in stimulating protein synthesis (in presence of ammonium salts) and increasing the rate of removal of 'high-energy phosphate bonds' was postulated, as this latter process was supposed to govern the rate of fermentation. Leijnse & Terpstra (1951), using a different technique of carboxylase estimation from that of Westenbrink *et al.* (1947), showed that the enzyme was formed on incubation of yeast for 4 hr., with either thiamine or 2-methyl-4-amino-5-ethoxymethylpyrimidine (hence the latter must have been converted into cocarboxylase), glucose and ammonium salts showing stimulatory actions.

In the previous paper (Trevelyan & Harrison, 1954) we have shown that, in fermentation by D.C.L. baker's yeast, a Michaelis-Menten equation may be used to describe the relation between fermentation rate and intracellular pyruvate level; and that one of the constants of this equation receives its simplest interpretation as the *in vivo* activity of the carboxylase system of the yeast cell. The present paper deals with the effect of thiamine, and of the pyrimidyl part of the thiamine molecule, on the fermentation rate-intracellular pyruvate relation. A diminution of pyruvate excreted by yeast into the medium, brought about by thiamine or cocarboxylase, has been reported by Haag & Dalphin (1940).

METHODS

Fermentation rate and pyruvate were determined as previously described (Trevelyan & Harrison, 1954). The yeast studied was again D.C.L. pressed baker's yeast. All experiments were carried out at 30°.

RESULTS

Effect of excess thiamine on pyruvate

These experiments were carried out using 20 or 30 ml. yeast suspension stirred by a stream of N_2, and contained in a 1 in. diameter Pyrex tube. In all cases 5% (w/v) yeast, and 0·1M glucose were used. Thiamine, when added, was 10^{-4}M, i.e. 2 μmoles/g. yeast. The effect of thiamine was to lower markedly both the extracellular and the intracellular pyruvate, as shown in Fig. 1. (It was immaterial whether the thiamine was added together with glucose at zero time, or had been previously added to the yeast, as in Fig. 1.) The effect was not dependent on the presence in the medium of ammonium salts, as Went (1949) claimed for the acceleration of fermentation rate, and low pyruvate values were obtained with yeast suspended in water (Fig. 2, A). When, after 30 min. fermentation in the presence of thiamine, the yeast was centrifuged, washed three times with water, and then used in a second fermentation, pyruvate values were still low (Fig. 2, B). In the experiment

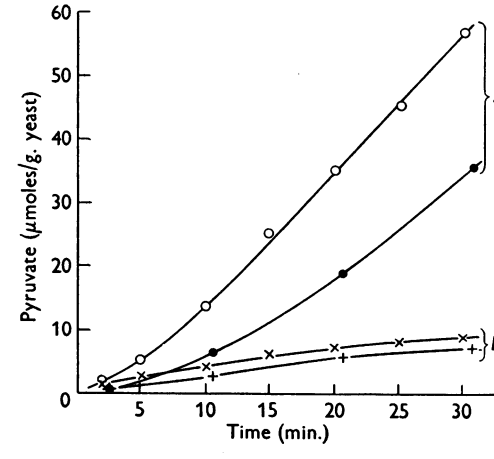

Fig. 1. Effect of thiamine (2 μmoles/g. yeast) on total and extracellular pyruvate in yeast fermentation. *A*, 5% (w/v) yeast; 0·1M glucose; 0·05M potassium succinate, pH 5·5; 0·005M-$(NH_4)_2HPO_4$. ○, total pyruvate; ●, extracellular pyruvate. *B*, as *A* with thiamine added. ×, total pyruvate; +, extracellular pyruvate.

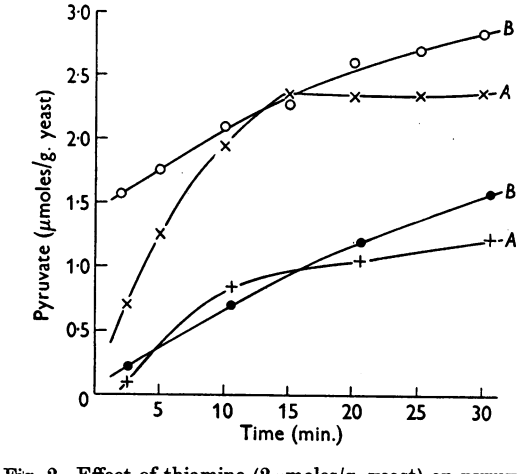

Fig. 2. Effect of thiamine (2 μmoles/g. yeast) on pyruvate during fermentation. *A*, unbuffered medium, pH 4·6. ×, total pyruvate; +, extracellular pyruvate. *B*, yeast previously allowed to ferment for 30 min. with thiamine and 0·1M glucose in potassium succinate, pH 5·5, then centrifuged and allowed to ferment in potassium succinate pH 5·5. ○, total pyruvate; ●, extracellular pyruvate.

of Fig. 3, the yeast was allowed to ferment for 32 min., to permit a high extracellular and intracellular pyruvate to be established, before the addition of thiamine (as 0·01 M solution). (Potassium succinate buffer was used as the medium, because pyruvate production between 10 and 30 min. had been found to be linear with time, hence the time course of pyruvate production before thiamine addition could be established using only two pyruvate determinations.) After addition of thiamine the production of extracellular pyruvate stopped, and a slight fall in pyruvate level set in. A dramatic fall in intracellular pyruvate followed thiamine addition, and was largely completed within 3 min. It is interesting to note that the highest extracellular pyruvate, at 35 min., was 1·0 μmole/ml. medium; the intracellular pyruvate fell to 1·8 μmoles/g. yeast, i.e. of the same order as the extracellular. Thus the effect of thiamine was clearly on the carboxylase system, either in (*a*) increasing the amount of carboxylase, (*b*) increasing the affinity of the enzyme for the substrate, or (*c*) increasing both. The rapidity of the effect made it unlikely that any increase in carboxylase could be due to synthesis of new protein. Cases (*a*) and (*b*) could in principle be distinguished by kinetic studies.

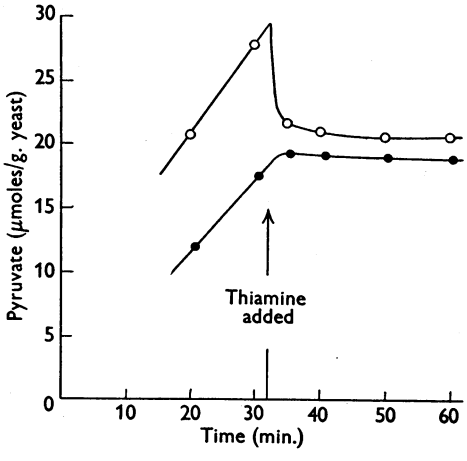

Fig. 3. Effect of thiamine (2 μmoles/g. yeast) added to established fermentation. 0·1 M glucose; potassium succinate buffer; pH 5·5. ○, total pyruvate; ●, extracellular pyruvate.

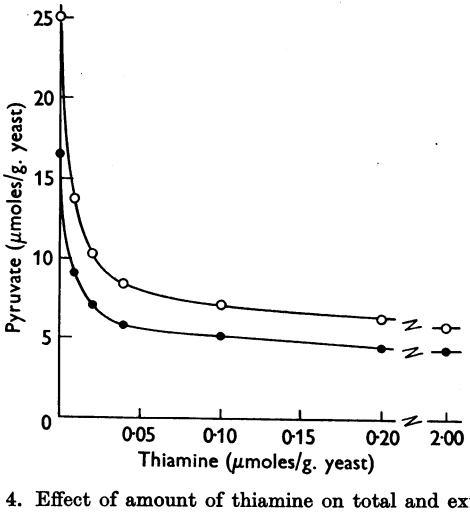

Fig. 4. Effect of amount of thiamine on total and extracellular pyruvate after 30 min. fermentation. 5% (w/v) yeast; 0·2 M glucose; sodium succinate buffer, pH 4·1; 0·01 M-KH_2PO_4; 0·01 M-$(NH_4)_2SO_4$; 0·0025 M-$MgSO_4$; 0·001 M-$ZnSO_4$; 0·001 M-$CaCl_2$; N_2 atmosphere. ○, total pyruvate; ●, extracellular pyruvate.

Effect on pyruvate of varying amounts of thiamine

The results are shown in Fig. 4. The medium used in the fermentations was that which gave the greatest pyruvate production in the absence of thiamine. An excess (2 μmoles/g. yeast) of thiamine caused a fall of 77 % in total pyruvate; the yeast was, however, nearly saturated by 0·1 μmole/g. which caused a 72 % drop, whilst as little as 0·01 μmole/g. gave a 45 % fall. It is probable that a method for estimating thiamine could be based upon the pyruvate effect.

Kinetics of the thiamine effect

In order to decide between the two possible effects of thiamine on the yeast carboxylase system, attempts were made to determine the effect of thiamine on the two constants $V_{max.}$ and K_m of the Michaelis-Menten type relation connecting fermentation rate and intracellular pyruvate (cf. Trevelyan & Harrison, 1954). Fermentations with D.C.L. baker's yeast, under the conditions used in this work, take some 35 min. to reach maximum rate and steady intracellular pyruvate: at this time the determination by difference of intracellular pyruvate becomes uncertain, especially in the presence of thiamine, owing to the relatively high extracellular pyruvate. Attempts to use the increasing rate in maltose systems, as described in the previous paper (Trevelyan & Harrison, 1954), failed, because the thiamine effect, though rapid, was sufficiently time-dependent to make determinations at various times over 4 hr. not comparable. It had been noted that if, after 35 min. fermentation the yeast was centrifuged and washed, and used in a second fermentation, then the internal pyruvate reached its maximum value in a very short time. It was therefore decided to determine the effect of a preliminary fermentation with thiamine for a standard time. The determination of $V_{max.}$ and K_m depended upon only two points, one of which

was derived from an arsenate-inhibited system. The arsenate was not added in the first fermentation, so that the yeast absorbed thiamine under nearly the same conditions in all experiments. (The only difference was in the omission from the medium of phosphate, which counteracts the inhibitory action of arsenate.) The first fermentation was for 35 min. at 30° when glucose was the substrate, and for 90 min. with maltose, thiamine being added for the last 30 min. only. The yeast was then centrifuged, washed once with medium at 30°, and made up to 100 ml. with medium at 30°: 50 ml., containing 5 g. yeast, were immediately taken for the main fermentation. An unexpected advantage of this technique was that a tendency to foam in the xylene extraction step of the pyruvate estimation was eliminated, allowing double the amount of material to be used, with a consequent improvement in accuracy in estimating the low pyruvate level of arsenate-inhibited fermentation.

The following is a summary of the intracellular pyruvate results. They were determined at 5 min. intervals, starting at the time stated. *Expt.* 1, glucose fermentation, no thiamine: (5 min.) 12·0, 13·3, 15·0, 14·5. *Expt.* 2, glucose fermentation, arsenate, no thiamine: (10 min.) 1·15, 1·09, 1·02, 1·04. *Expt.* 3, glucose fermentation, 0·01 μmole thiamine/g. yeast: (5 min.) 3·9, 5·3, 5·9, 5·8. *Expt.* 4, glucose fermentation with arsenate, 0·01 μmole thiamine/g.: (10 min.) 0·78, 0·71, 0·69, 0·66. *Expt.* 5, maltose fermentation, 0·01 μmole thiamine/g.: (5 min.) 1·31, 1·30, 1·45, 1·46. *Expts.* 6 and 7 concerned the effect of higher thiamine concentrations, only the uninhibited glucose fermentation being studied. Pyruvate values at 10 and 20 min. were: *Expt.* 6, 0·02 μmole thiamine/g., 3·18, 2·74. *Expt.* 7, 0·10 μmole thiamine/g., 1·85, 1·39. Rate determinations were based on the CO_2 evolved over 10 min. intervals. In glucose fermentation they varied little after the first 10 min. The failure to observe the increasing fermentation rate in the presence of thiamine, described, e.g. by Schultz *et al.* (1937*a*, *b*), is due to the high yeast concentration used. In calculating the constants of the Michaelis-type equation the following points were used: for high-rate fermentations (Expts. 1, 3), mean pyruvate and fermentation rate at 10, 15 and 20 min., the rate at 15 min. being found by graphical interpolation; for arsenate fermentation (Expt. 4), mean pyruvate values at 15, 20 and 25 min. Mean values for the single maltose fermentation (Expt. 5) were used only as a check point.

Incubation with 0·01 μmole thiamine/g. does not alter the K_m value (Fig. 5): the values of 2·9 and 3·1 μmoles pyruvate/g. are the same as previously obtained (Trevelyan & Harrison, 1954). The maximum velocity $V_{max.}$, however, increases from 71 to 103 μmoles CO_2/min./g. From Expts. 3, 6 and 7 it appears that increasing the amount of thiamine does not affect the actual fermentation rate observed (medium as under Methods, Trevelyan & Harrison, 1954), hence in Fig. 5 all the thiamine points lie on a horizontal line. Assuming that $K_m = 3$ μmoles/g. in all cases, then 0·02 μmole thiamine/g. corresponds to a $V_{max.}$ of 135 μmoles CO_2/min./g., whilst saturating the yeast with 0·10 μmole thiamine/g. gives $V_{max.} = 192$. The change in $V_{max.}$ due to 0·02 μmole thiamine/g., viz. 64, is double that induced by 0·01 μmole/g.

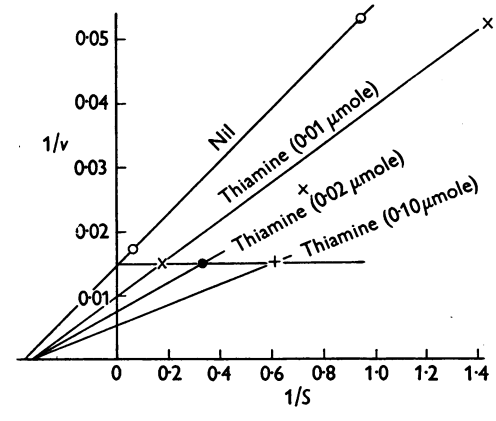

Fig. 5. Michaelis plot. v = fermentation rate; S = intracellular pyruvate.

Table 1. *Thiamine formation from 2-methyl-4-amino-5-ethoxymethylpyrimidine, calculated from pyruvate effect*

Time after adding pyrimidyl (min.)	Intracellular pyruvate S (μmoles/g.)	CO_2 rate v (μmoles/min./g.)	$\left[=v\left(1+\frac{3}{S}\right)\right]$ ($V_{max.}$) (μmoles CO_2/min./g.)	$\Delta V_{max.}$* (μmoles CO_2/min./g.)	Thiamine* $\left(=\frac{\Delta V_{max.}}{3200}\right)$ (μmoles/g.)
5	11·3	58·2	74	3	0·0009
15	7·2	65·4	93	22	0·0069
25	5·9	66·0	100	29	0·0091
35	4·7	66·1	108	37	0·0115
45	3·9	64·6	114	43	0·0134

* Calculated by taking $V_{max.}$ as 71 μmoles CO_2/min./g., and $\Delta V_{max.}$ at a value of 32 μmoles CO_2/min./g. as equivalent to 0·01 μmole thiamine/g.

In Expt. 8, 0·02 μmole thiamine/g. was added only after fermentation with pretreated yeast had proceeded for 12 min. The pyruvate values at 10 min., i.e. before thiamine addition, were only 5% different from those of Expt. 1; 30 min. after thiamine addition the intracellular pyruvate was the same as in Expt. 6, where thiamine was present during the preliminary fermentation. The time course of the effect of 0·02 μmole thiamine/g. in Expt. 8 may be approximately translated into changes in $V_{max.}$ by applying the pyruvate values to the horizontal line of Fig. 5. Three minutes after thiamine addition $\Delta V_{max.} = 32$ μmoles CO_2/min./g.; after 8 min., 49; after 18 min., 52; after 30 min., 59.

Addition of the pyrimidyl part of the thiamine molecule (2 μmoles/g. yeast) also produced pyruvate changes, but the fall in pyruvate proceeded more slowly. The pyruvate changes may be transformed into $\Delta V_{max.}$ values, and using the relation that $\Delta V_{max.}$ of 32 μmoles CO_2/min./g. is equivalent to 0·01 μmole thiamine/g., the approximate amounts of thiamine apparently synthesized from 2-methyl-4-amino-5-ethoxymethylpyrimidine may be calculated; details of the calculation are summarized in Table 1.

DISCUSSION

The results support the view of Leijnse & Terpstra (1951) that thiamine, added during yeast fermentation, actually increases the amount of the enzyme carboxylase. The rapidity of its effect, however, suggests that with the yeast we have used it does so by supplying coenzyme to pre-existing apoenzyme, at least in the first 10 min. or so. Whether such formation of new carboxylase is reflected in an increased fermentation rate depends on carboxylase being the sole limiting enzyme (cf. Trevelyan & Harrison, 1954) for the yeast used. For the yeast studied here the fastest rate so far observed was 63 μmoles CO_2/min./g.; the $V_{max.}$ value for the carboxylase system was only 71, hence carboxylase activity could be said to limit fermentation rate. Thiamine, from 0·01 to 2·00 μmoles/g., gave a rate of 73; though $V_{max.}$ was increased up to about 200. This implies that another enzyme system now imposes a limit of 73 on the observed rate of CO_2 production. If amounts of thiamine over about 0·005 μmole/g. are to influence the fermentation rate, synthesis of non-carboxylase protein may well be required. It is obvious that different strains of yeast may react differently in regard to such factors as incubation time or presence of assimilable nitrogen.

We have seen that incubation of yeast for 30 min. with 0·01 μmole thiamine/g. increases $V_{max.}$ by 32 μmoles CO_2/min./g. Since the original $V_{max.}$ is 71, then the cocarboxylase content of the original yeast ought to be about 0·02 μmole/g. Actually the type of yeast used contains, by direct thiamine estimation, 0·01–0·02 μmole 'combined' thiamine per gram. Also, 1 mole cocarboxylase, according to these figures, catalyses the production of some 3000 moles CO_2/min. Jansen (1949) quotes a figure of 1500, based on the reconstitution of carboxylase in alkali-washed yeast. Green, Herbert & Subrahmanyan (1940, 1941) gave a value of 840 for their purified yeast enzyme.

There seems little doubt that the action of the pyrimidine part of the thiamine molecule depends upon its prior conversion into thiamine (cf. Leijnse & Terpstra, 1951). From Table 1 the rate of synthesis would appear to be some 3×10^{-4} μmole/min./g., which means that the thiamine content of the yeast would double after about 1 hr. Rates of a similar order for synthesis and degradation of coenzymes in various types of micro-organism have been reported by McIlwain (1947).

SUMMARY

1. Thiamine, added to fermenting baker's yeast, lowers the intracellular pyruvate corresponding to a given fermentation rate.

2. Kinetic studies suggest that this is caused, not by increased affinity of carboxylase for its substrate, but by the production of more enzyme.

3. The rapidity of action is interpreted as showing that, at least in the early stages, actual protein synthesis is not involved, i.e. that the process is one of supplying coenzyme to pre-existing apocarboxylase.

4. The pyruvate-lowering action of thiamine increases when 0·01–0·10 μmole/g. yeast is added, but the fermentation rate does not parallel thiamine increase above 0·01 μmole/g. It is pointed out that effects on CO_2 rate may depend upon the enzymic constitution of the particular yeast used.

5. The pyruvate-lowering action of 2-methyl-4-amino-5-ethoxymethylpyrimidine is explained as due to the synthesis from it of 3×10^{-4} μmole thiamine/min./g. yeast.

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The Estimation of Purines in Nucleic Acids: a Method Applicable to Materials with Low Concentration of Nucleic Acid

By I. W. McDONALD
Agricultural Research Council, Institute of Animal Physiology, Babraham Hall, Cambridge

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During the course of studies on the metabolism of protein in the rumen of sheep, it became desirable to obtain an estimate of the content of nucleic acids in the digesta; this material consists of a heterogeneous mixture of plant material and a wide variety of bacteria and protozoa. No success was achieved in attempting to apply the procedures based on phosphorus partition (Schmidt & Thannhauser, 1945), colorimetric tests for pentoses (Schneider, 1945) or separation of the purines as the copper complexes (Graff & Maculla, 1935; Vendrely & Sarciron, 1944). Direct spectrophotometry, as used for example by Mitchell (1950), or chromatography, as developed by Markham & Smith (1949), could not be applied owing to the low concentration of nucleic acid in the materials analysed. No procedure has yet been devised for the quantitative separation of the nucleic acids from plant material. It was therefore decided to estimate the nucleic acids in terms of the purines, adenine and guanine; this was achieved by mild acid hydrolysis followed by precipitation of the purines as the silver salts (Schmidt & Levene, 1938; Gulland, Jordan & Threlfall, 1947), the separation of the two purines by chromatography (Markham & Smith, 1949) and final estimation by spectrophotometry. The method has been applied to preparations of protozoa and bacteria, plant leaves and other feeding stuffs as well as to digesta.

PROCEDURE

Preparation of sample. Before hydrolysis it is necessary to remove chloride and purine-containing compounds other than nucleic acids. Samples containing about 1 mg. of purine-N were extracted with 80% (v/v) ethanol containing approximately 0·001 N-H_2SO_4, in the case of dry samples, or treated with absolute ethanol to a final concentration of 80% in the case of aqueous suspensions.

The mixture was filtered with gentle suction over a thin layer of Celite, in a sintered glass funnel, and the precipitate was washed twice with 80% ethanol, once with absolute ethanol and once with acetone. The major part of the acetone was removed by suction and the last traces by drying the filter in an oven at low temperature.

Hydrolysis. The residue was hydrolysed in 17 ml. of N-H_2SO_4 in the boiling water bath for 1 hr. When handling coarse, fibrous plant material, considerable care was required at this stage. To prevent charring of polysaccharides the hydrolysis was performed in glass-stoppered tubes; the dry filter cake was thoroughly wetted with a little of the acid and transferred to the hydrolysis tube; the remainder of the acid was used to complete the quantitative transfer of the sample. At first, the mixture was cautiously warmed in the bath until occluded air was removed; the glass stopper was then inserted and the hydrolysis allowed to proceed.

In order to avoid errors due to change in volume of the acid during hydrolysis, the hydrolysate was separated from the bulky fibrous residue by straining through a disk of fine stainless steel gauze fitted in the bottom of an all-glass syringe; the fluid was expressed with the aid of compressed air and the pad compressed with the glass piston; the fibrous pad was washed in a similar manner several times with water. The completeness of washing could readily be verified by titration of residual acid in an aqueous extract of the fibre pad. The filtrate and washings were collected into a 25 ml. flask, made to volume and filtered through Whatman no. 42 paper.

Separation of the silver-purines. To 20 ml. of the filtrate was added 1 ml. of 0·5 M-$AgNO_3$ and the mixture was chilled in the refrigerator for 1–2 hr.; the inner wall of the centrifuge tube was then carefully rubbed down with a rubber-tipped glass rod to loosen any adherent precipitate. The rod was rinsed down with about 1 ml. of saturated Ag_2SO_4, in 0·01 N-H_2SO_4. After chilling again, the tubes were centrifuged at 0°, the supernatant fluid was removed and the wall of each tube carefully washed down with a small volume of the Ag_2SO_4 solution; this washing was best performed with a coarse Pasteur pipette carrying a short