addition of sufficient amounts of DPN to mitochondrial preparations restores almost completely pyruvate oxidation under aerobic conditions[2]. It may be remembered, with regard to the last point, that the effect of the addition of TPP on the oxidation of pyruvate by oxygen is also very small in homogenates of breast and heart muscles of thiamine-deficient pigeons (FRANKEN AND STAPERT[12]).

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SUMMARY

The distribution of TPP in cytoplasm fractions of rat liver is studied. When the homogenate was prepared with 0.25*M* sucrose, about 30% of TPP was present in mitochondria. When the homogenization medium was distilled water, the amount of TPP present in this fraction was markedly decreased, while that present in the supernatant fluid was increased. Mitochondria suspended in 0.25*M* sucrose and incubated at 18° C for 10 min release in the suspension fluid about 20% of their TPP; under the same conditions, mitochondria suspended in water release about 65% of their TPP. An increased destruction of TPP was observed as a result of incubation of mitochondria in water at 18° C.

The distribution of TPP in fatty liver homogenates prepared in 0.25 *M* sucrose resembles strongly that observed for homogenates of normal rat liver prepared in distilled water; TPP was decreased in the mitochondrial fraction and correspondingly increased in the supernatant. Total TPP is decreased in fatty liver obtained by feeding rats on a diet deficient in choline. It remains practically unchanged in fatty liver produced by CCl_4.

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THE EFFECT OF EDTA ON THE INTERACTION BETWEEN ACTOMYOSIN AND ADENOSINE TRIPHOSPHATE*

YUJI TONOMURA, HIROYUKI MATSUMIYA AND SHOTARO KITAGAWA

Research Institute for Catalysis, and Chemistry Department, Faculty of Science, University of Hokkaido, Sapporo (Japan)

It has been well established by FRIESS[1] and BOWEN AND KERWIN[2] that EDTA is a striking activator of myosin ATPase in the presence of high concentration of KCl.

* The following abbreviations will be used; ATP adenosine triphosphate, ATPase adenosine-triphosphatase, and EDTA ethylenediaminetetraacetic acid.

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In addition, FRIESS *et al.*[3] have demonstrated that this acceleration is mediated by a metal ion (probably Mg^{++}) fixed to the protein structure. In a recent paper, further suggestion was made by KIELLEY AND BRADLEY[4] that sulfhydryl groups are involved in the EDTA activation. They[5] have also observed a marked increase of the Michaelis constant of myosin ATPase in the presence of K^+–EDTA. On the other hand, EDTA has an inhibitory action on the decrease of viscosity and intensity of light scattered by actomyosin solution after the addition of ATP, as was shown by several investigators[1,6–8].

However, these investigations appear to deal with rather qualitative aspects[6,8] or to be performed at a fixed concentration of EDTA[5]. In the present paper, therefore, the effect of EDTA on actomyosin ATPase and the change in light scattered by actomyosin solution caused by ATP was measured over a wide range of concentrations of EDTA (0–20 m*M*), and analyzed quantitatively. In addition, the transitions, in the presence of EDTA, of both the change of the physical state of actomyosin after ATP addition and the recovery from the change could be separately recorded with a recording apparatus using the light-scattering method.

EXPERIMENTAL

Materials

Myosin B (natural actomyosin) was prepared from rabbit skeletal muscle according to standard practice in this laboratory[9]. ATP was prepared as previously described[9] and purified on a column of Dowex-1 by means of the usual chloride cycle elution[10]. EDTA was obtained commercially and used as the sodium salt (pH 7.0).

Procedures

The ATPase activity was determined by measuring orthophosphate liberated according to the FISKE-SUBBAROW method[11]. The usual incubation mixture contained 0.03 *M* tris-maleate buffer at pH 6.7 and various concentrations of EDTA with 0.6 *M* KCl. Incubations were carried out for several periods ranging from 0 to 10 min at 23° C. In some experiments deviation in the concentration of Na^+ owing to use of highly concentrated Na^+-EDTA was adjusted by adding the appropriate amount of NaCl solution to the reaction mixture of EDTA of lower concentration. The dephosphorylation rate was unaffected within experimental error, irrespective of whether this adjustment was adopted or not.

The grade of the light-scattering drop (see below) was determined at 23° C and pH 6.7 by a method essentially identical to the one reported in a previous paper[12]. In order to trace the transition of light scattered by myosin B solution, the current output of the photomultiplier was amplified with an electrometer tube according to ITOKAWA[13] and recorded by means of an electromagnetic oscillograph (YEW, 6 elements, oscillator D type). In this procedure, the accuracy in time was dependent on the rapidity of mixing ATP or EDTA with myosin B solution. By blowing out a measured amount of ATP or EDTA solution, using a pipette with its tip cut rectangularly to its axis, it was easy to reduce the mixing time to less than 1 sec.

The content of protein was calculated by multiplying by a factor of 6 the nitrogen content determined by the micro Kjehldahl method.

RESULTS

Adenosinetriphosphatase

As is well known, the relation between the reaction velocity (v) of myosin B ATPase and the concentration of ATP ([S]) is given by the Michaelis-Menten formula[9,14]:

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}}$$

The Michaelis constant (K_m) and the reaction velocity at sufficiently high concen-

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tration of substrate (V_{max}) in the presence of EDTA was determined according to the LINEWEAVER-BURK procedure[15], and the results are summarized in Table I. As already reported by KIELLEY *et al.*[5], both K_m and V_{max} increased remarkably with increasing concentration of EDTA. It is to be noted that when EDTA concentration exceeded 5 mM, v became almost independent of the EDTA concentration and, moreover, the line $1/v$ versus $1/[S]$ was found to pass through the origin, showing measurelessly high values of K_m and V_{max}. The first order velocity constant of ATPase, V_{max}/K_m, *i.e.* the velocity constant at low concentration of ATP, decreased only slightly by the addition of EDTA, because K_m and V_{max} were simultaneously enlarged.

TABLE I

EFFECT OF EDTA ON MYOSIN B ATPASE

K^+, 0.6 M; Na^+, *ca.* 0.05 M, pH 6.7; 23° C.

Concentration of EDTA (mmoles/l)	V_{max} *(μmoles/g/sec)*	K_m *(moles/l)*	V_{max}/K_m *(l/g/sec)*
0	2.8	$3.6 \cdot 10^{-4}$	$7.6 \cdot 10^{-3}$
0.5	4.35	$6.2 \cdot 10^{-4}$	$7.0 \cdot 10^{-3}$
5, 10, 20	< 100	$< 1 \cdot 10^{-2}$	$5.4 \cdot 10^{-3}$

Grade of light-scattering drop

The grade of the drop in light scattering (Δ) is defined by: $\Delta = (I_0 - I_s)/(I_0 - I_\infty)$, where I_0 and I_s represent the 90° scattered intensities before and after the addition of a certain quantity of ATP, and I_∞ is the intensity with a sufficiently large quantity of ATP. Then, Δ may represent the molar ratio of the physically changed myosin B to the total myosin B (see appendix of ref. [12] and ref. [16]). The relation between Δ and the concentration of ATP added ([S]) was given by a dissociation curve of the first order in the absence of[16,17] and at a low concentration of EDTA (Fig. 1). That is,

$$\Delta = \frac{1}{1 + \frac{K}{[S]}} \quad \text{or} \quad \Delta = \frac{1}{2} + \frac{1}{2} \tanh\left(2.303 \frac{1}{2} \log \frac{[S]}{K}\right).$$

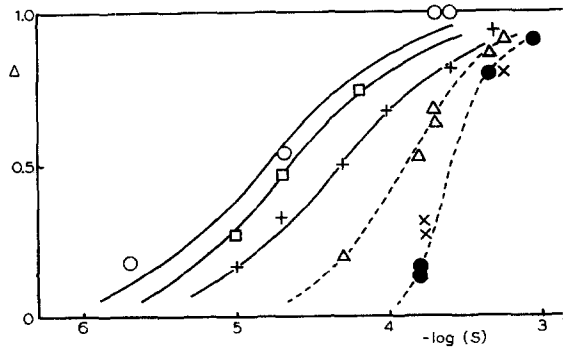

Fig. 1. Relationship between grade of drop of the light scattering (Δ) and the concentration of ATP added ([S]). Concentration of myosin B, 1 mg/ml; K^+, 0.6 M; Na^+, *ca.* 0.05 M, pH 6.5; 23° C. EDTA concentration; ○, 0; □, 1 mM; +, 2.5 mM; △, 5.0 mM; ×, 10 mM; ●, 19–20 mM. The solid lines correspond to $\Delta = 1/2 + 1/2 \tanh (2.303\ 1/2 \log [S]/K)$.

However, the order of the relation Δ – [S] was raised gradually by enhancement of EDTA concentration, and when concentration of EDTA was higher than 5 mM, it became approximately second. The apparent dissociation constant K, corresponding

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to [S] necessary for 50% Δ, was enlarged with increasing EDTA concentration, and reached a constant value in the neighbourhood of 10 mM EDTA, when the correction due to breakdown of ATP during the drop phase was made.

Velocity of light-scattering drop

The velocity of the drop of light scattered by myosin B solution after the addition of ATP fell off strikingly under the influence of EDTA (Fig. 2). At the initial state, where the recovery process can be neglected, the rate of change in the scattered intensity can be given by the following formula:

$$\frac{d[M^*]}{dt} = -\frac{d[M]}{dt} = k_{drop}[M]$$

where [M] and [M*] represent the concentration of the original and the physically changed myosin B, respectively, and k_{drop} is the velocity constant of the drop, provided that [S] is constant. As has been demonstrated by BÁRÁNY *et al.*[18] and one of the present authors[19], when the quantity of ATP is so large that its adsorption to and decomposition by myosin B can be neglected, a linear relation is obeyed between the logarithm of the light-scattering drop and the reaction time, *i.e.*, $-2.303 \log (1-\Delta) = k_{drop}\, t$. Although this relationship did not hold good in the presence of EDTA owing to decomposition of ATP* and coexistence of the recovery process, the approximate value of k_{drop} could be calculated from the slope of $\log (1-\Delta)$ versus t, as shown in Fig. 2. The calculated values of k_{drop} by means of this method were 1/18–1/19 and 1/10–1/11 sec^{-1} in the presence of 19 mM EDTA and at 0.635 and 1.21 mM ATP, respectively**. These values are remarkably lower than that observed in the absence of EDTA (about 1 sec^{-1} [9]). In addition, it was found that the transition of the light-scattering drop caused by adding ATP immediately after the addition of EDTA, was identical with that observed when ATP was added several hours after incubation of the reaction mixture with EDTA. It seems, therefore, that the equilibrium between EDTA and the myosin B-ATP system is accomplished instantaneously.

Velocity of recovery step

Usually, the recovery process from the decreased intensity follows an S-shaped curve, because it accompanies the breakdown of ATP[20]. Therefore, the record of the recovery process under ordinary conditions can give little information about the mechanism of the underlying change of actomyosin (M* $\longrightarrow$ M). But, since the equilibrium between EDTA and myosin B is achieved rapidly (see above), the recovery step itself (M* $\longrightarrow$ M) may be measured, separating it from the decreasing phase, when a large amount of EDTA, sufficient to prevent the occurrence of the drop phase entirely, is added at the reduced state of the light scattering. In Fig. 3 is inserted a typical oscillogram of the recovery step obtained by the addition of 19 mM EDTA to myosin B solution at the reduced state, which had been brought about by the addition of $8 \cdot 10^{-5} M$ ATP. As shown in the figure, the plot of $\log \Delta$ against t followed

* In the presence of 1 mg/ml of myosin B and 20 mM EDTA, about 6% of ATP was decomposed during 10 sec.

** The authors have not determined dependence of k_{drop} on [S], because k_{drop} can be measured only very roughly at a range of low [S].

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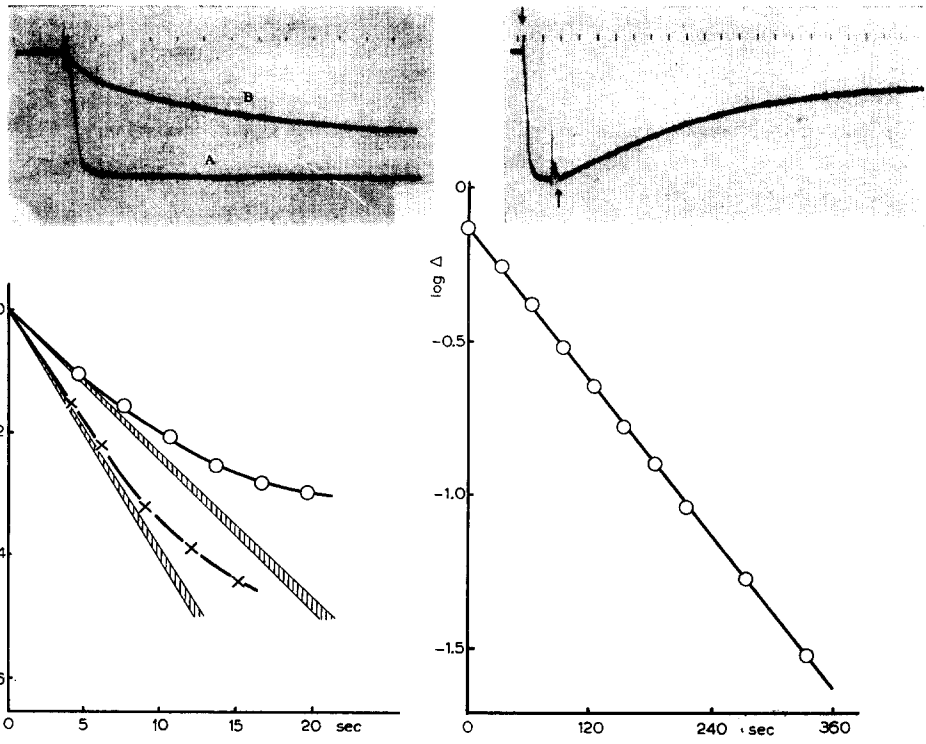

Fig. 2. Drop of the scattered intensity as a function of time after ATP addition. Correction for a dilution by ATP addition was made. ○, ATP concentration, 0.635 mM; EDTA concentration, 19 mM. ×, ATP concentration, 1.21 mM; EDTA concentration, 19 mM. Other experimental data as in Fig. 1. The upper figure is inserted as examples of oscillograms of the light-scattering change following the addition of 0.635 mM ATP. One time-scale per 3 sec. A; EDTA concentration 0. B; EDTA concentration, 19 mM.

Fig. 3. Recovery from the reduced scattering intensity as a function of time after EDTA addition, calculated from the upper oscillogram. One time scale per 12 sec. Arrow down, addition of $8 \cdot 10^{-5} M$ of ATP; arrow up, addition of 19 mM of EDTA. Other experimental data as in Fig. 1.

strictly a straight line. Thus, the mechanism of the recovery step may be given as follows:

$$-\frac{d\,[M^*]}{dt} = k_{rec}\,[M^*]$$

whence

$$-2.303 \log \Delta = k_{rec}\, t,$$

where k_{rec} denotes the velocity constant of the recovery step. The value of k_{rec} obtained from the slope of the line $\log \Delta - t$ was $1/105$ sec^{-1}. In the presence of $8 \cdot 10^{-5} M$ ATP, the recovery step caused by the addition of 10 mM EDTA followed quite the same time course as that of the above one. However, on the addition of 5 mM EDTA, which is not sufficient to counteract the action of ATP (see Fig. 1), considerable reduction in the rate of the recovery was observed, as was expected.

Ratio of rates of decreasing step to recovery step

From the value of k_{drop} and k_{rec} determined in the previous sections the ratio between

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these two constants, in the presence of 19 mM EDTA, was calculated and found to be 9.5–10.5 and 5.5–6 at 1.21 and 0.635 mM ATP, respectively. On the other hand, as given in Fig. 2, $\Delta/(1-\Delta)$, which may be equal to the molar ratio of M* to M, was observed to be $\sim$9 and $\sim$4 in the presence of 0.9 and 0.48 mM ATP*, respectively. Thus it may be said that the ratio k_{drop} to k_{rec} coincides approximately with the molar ratio of the physically changed myosin B to that of the original myosin B.

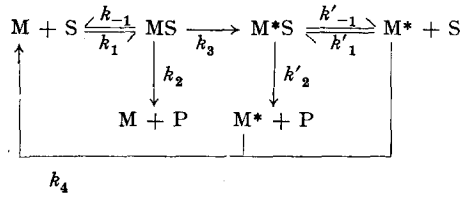
DISCUSSION

The effect of EDTA on myosin B ATPase in 0.6M KCl can be ascribed to acceleration of the decomposition of the enzyme-substrate complex as shown typically by measurelessly high values of V_{max} and K_m. Such a concomitant increase of K_m and V_{max} was not observed in the case of the activation by Ca^{++}, which has been found to enhance V_{max} without marked effect on K_m[14,21]. Although the relation between ATPase and the optical change is not fully clarified, there are some indications that the optical change is the result of the combination of ATP with the ATPase active site of myosin[16,17,22]. Based on this probable assumption, two alternative mechanisms for the myosin B-ATP system may be formulated. The one, which is implicitly adopted by several authors, is written as follows (*cf.* ref. [23]):

$$M + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} M^*S \xrightarrow{k_2} M + P$$

where k_1 is the velocity constant of each reaction. If this mechanism is correct, k_{rec} must be equal to $k_2 + k_{-1}$. As described above, V_{max} in the presence of 19 mM EDTA was larger than 100 μM g^{-1} sec^{-1}. Since the weight unit for the ATP binding site is $2\text{–}4 \cdot 10^5$ [9,24], k_2 becomes greater than 20–40 sec^{-1} ($100 \cdot 10^{-6} \times 2 \cdot 10^5 - 100 \cdot 10^{-6} \times 4 \cdot 10^5$), which is several thousand times greater than k_{rec}. Therefore, it seems obvious that this scheme is hardly applicable for a reaction mechanism, at least in the presence of EDTA.

On the other hand, BLUM[16] and TONOMURA[9,17] have proposed the following scheme:



The inhibitory effect of EDTA on the optical change of myosin B caused by ATP may reasonably be explained by this scheme. That is, EDTA makes the intermediate complex (MS) in the physical change very unstable (striking enhancement of k_2). Accordingly, almost all of the intermediate formed is decomposed directly to M + P without passing through the dissociation[25,26] or the deformation step[20] (MS $\longrightarrow$ M*S).

In the presence of EDTA, the majority of myosin B molecules exist as M or M* in the reaction medium because of the unstability of the enzyme-substrate complexes.

* A correction for breakdown of ATP was made.

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It follows, therefore, that

$$k_{\text{drop}} \sim k_3 \frac{[S]}{K_m}$$

$$k_{\text{rec}} = k_4 .$$

And at the steady state,

$$\frac{1-\Delta}{\Delta} \sim \frac{[M]}{[M^*]} = \frac{k_4}{k_1\left(\frac{k_3}{k_2+k_{-1}+k_3}\right)[S]}$$

$$\sim \frac{k_4}{k_3\left(\frac{k_1}{k_2+k_{-1}}\right)[S]} = \frac{k_4 K_m^*}{k_3 [S]} ,$$

that is,

$$K \sim \frac{k_4}{k_3} K_m \text{ and } \frac{[M]}{[M^*]} \sim \frac{k_{\text{rec}}}{k_{\text{drop}}} .$$

Thus, as was found experimentally (see above), the ratio of the rate of the recovery step to that of the decreasing step is almost the same as the molar ratio of the original protein to the physically changed one, and, in addition, it may be concluded that the very small value of the optical K compared to the chemically derived K_m is due to the small value of the ratio k_4/k_3. But the second order relation between Δ and [S] found in high concentration of EDTA remains unexplained by the above scheme. The actual mechanism of the change may be given as follows:

$$M + S \underset{(1)}{\rightleftharpoons} MS + S \underset{(2)}{\rightleftharpoons} MSS \longrightarrow M^*SS \longrightarrow$$

and the equilibrium of step (1) may shift to the left at high concentrations of EDTA**.

As described above, the rate-limiting step of the recovery from the physically changed myosin B to the original one undoubtedly obeyed a monomolecular reaction. Although this holds true in the presence of EDTA, it may also occur in the absence of EDTA. Therefore, our result seems to exclude the possibility, at least in the presence of EDTA, that the combination of actin and myosin represents the rate-determining step in the recovery process, and it supports the view proposed by TSAO[27] that ATP appears to cause dimerisation of the actin partner in actomyosin and that the rate of the recovery process is determined by re-monomerisation of the actin partner.

But, of course, these kinetic deductions do not necessarily form an objection to the dissociation theory[25]. Recently, a preliminary study[28] on the rate of formation of actomyosin was made by means of tracing increase of scattered intensity after

* In the case of EDTA present, the relation $1/v$ versus $1/[S]$ was found to follow a straight line, even when the physically changed protein coexisted with the original one. Then,

$$K_m = \frac{k'_2 + k'_{-1}}{k'_1} = \frac{k_2 + k_{-1}}{k_1} .$$

** Another possible explanation of this sort of deviation for a multivalent macromolecular system was suggested by BLUM[16]. If EDTA increases an internal "viscosity" or "stability" of actomyosin molecule, then in the presence of EDTA and at low substrate concentration the shape or size change might be less than expected for a given set of kinetic constants.

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mixing F-actin (0.2 mg/ml) with myosin (0.8 mg/ml). The time interval required for 50% formation of actomyosin was found to be about 10 sec in the presence of 20 mM EDTA. The value is considerably less than the time necessary for 50% recovery from the changed state under almost identical experimental conditions, *viz.* 2.303 log 2 $\times$ 105 = 73 sec. The time-course is not yet exactly determined and it is not clear whether this discrepancy is due to the substantial difference between the reaction mechanism of the recovery step and the formation of actomyosin or to the difference between the nature of myosin B and synthetic actomyosin. This point must be the subject of future research.

FRIESS *et al.*[3] have concluded that the activating effect of EDTA on myosin ATPase seems to be mediated by Mg^{++} intrinsically bound to the protein and KIELLEY *et al.*[4] have suggested that SH groups are involved in this binding. According to the above experiments, saturation of the effect of EDTA on ATPase and on the optical change is not observed until the concentration of EDTA is raised relatively high (5–10 mM). This result suggests rather loose binding of EDTA with the intrinsic Mg^{++}. Besides incomplete chelation of EDTA to that metal, this rather loose binding seems to be attributable to a large repulsive force between ATP and EDTA as a result of the attachment of these anions on sites situated very close to each other on the protein molecule.

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SUMMARY

1. In the presence of 0.6M of KCl, EDTA increases concomitantly the V_{max} and K_m of myosin B ATPase. Especially when concentration of EDTA is higher than 5 mM, the line $1/v$ versus $1/[S]$ passes through the origin.

2. EDTA decreases both grade and rate of the optical change of myosin B caused by ATP addition.

3. The recovery step from the physically changed myosin B to the original myosin B follows strictly monomolecular kinetics.

4. On the basis of these results the reaction mechanism of EDTA with the myosin B-ATP system is discussed.

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NUCLEIC ACID METABOLISM OF THE DEVELOPING CHICK EMBRYO

II. DESOXYRIBONUCLEIC ACID AND CELL DIVISION

D. H. MARRIAN, A. F. PHILLIPS AND S. M. WERBA
Department of Radiotherapeutics, University of Cambridge (England)

INTRODUCTION

For some time, it was generally believed that the desoxyribonucleic acid (DNA) of a mammalian cell was not retained during cell division, so that two units of DNA had to be newly synthesised[1–5]. More recent work, with arguments derived from cell counts and uptake of labelled phosphate, has not confirmed this view[6–9], thereby bringing mammalian division into line with bacterial division, where the original DNA is retained[8, 10]. The present paper shows that the purine fragments of the DNA of the chick embryo are also retained during development.

METHODS AND MATERIALS

Injection of labelled precursors. Injections of sodium ^{14}C-formate and 2-^{14}C-glycine into fertile eggs were carried out as described previously[11].

Counting techniques

A change in spreading technique has led more certainly to "infinitely thin" conditions. After drying off the solution of the purines in dilute formic acid (pH 2), glass-distilled water (enough to cover the bottom of the planchette) is added, dried off, and the cycle repeated. Counts per aliquot of the original solution are much more consistent and this modification, together with a slight change in planchette size, has led to an increase in the counting rate by a factor of 1.19

References p. 583.