STUDIES ON PROTEIN SYNTHESIS WITH RIBONUCLEOPROTEIN PARTICLES FROM PEA SEEDLINGS

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(Received October 7th, 1958)

SUMMARY

Ribonucleoprotein particles from pea seedlings were isolated by conventional techniques and incubated in phosphate buffer with Mg, ATP and radioactive phenylalanine.

It was found that a substantial amount of label was incorporated into protein and at the same time a net increase in the amount of protein, amounting to about 250 μg per mg original protein, was observed.

The effects of ATP and chloramphenicol on the synthesis of protein as well as the effect of storing the particles prior to the incubation were also studied.

INTRODUCTION

Ribonucleoprotein particles from pea seedlings are particularly easy to obtain in a relatively homogeneous state by simple centrifugation, without treatment with desoxycholate[1,2]. For this reason they are a good material for studying the incorporation of labeled amono acids, *in vitro*.

The crude particles have the further advantage over those prepared from other sources, particularly liver, of being rather self-sufficient; *i.e.*, they can incorporate labeled amino acids without the addition of extraneous factors other than ATP, Mg and the labeled amino acid. Particularly, it has been shown by WEBSTER[3] and by CLARK[4] that these particles carry with them enough amino-acid-activating enzymes to obviate the addition of the supernatant fraction. Since the total concentration of protein can thus be kept very low, there is a good chance of detecting small changes in the net amount of protein occurring during the experiments.

On the other hand, this self-sufficiency makes it difficult to determine the requirements of the amino-acid-incorporating system. The data reported so far are indeed somewhat conflicting and it was partly in the hope of clarifying some of these apparent conflicts that the present investigation was undertaken.

MATERIALS AND METHODS

Seeds of *Pisum sativum*, var. "Alaska" were soaked in 0.5 % sodium hypochlorite for 10 min, washed with distilled water and planted in wet vermiculite. They were allowed

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to germinate under red light at 26° and 90% relative humidity for periods of time varying from 3 to 7 days[6]. With young seedlings, up to 5 days of age, only the cotyledons were removed prior to homogenization; with 7 days old seedlings, only the stems were used.

The seedlings were ground in an ice-cold mortar with an equal weight of cold sand and a volume corresponding to 0.5 to 1.0 times their weight of cold 0.1 *M* potassium phosphate buffer containing 0.45 *M* sucrose, at a pH of 7.1 or 7.5. These conditions are essentially those employed in the early experiments of WEBSTER[1,6].

The ground material was filtered through cheese cloth and centrifuged for 10 min at 2,000 rev./min (500 × *g*) in a clinical centrifuge. The supernatant, designated as "whole extract" was further fractionated in a Spinco ultracentrifuge, model L, according to the scheme in Fig. 1. After centrifuging at 10,000 × *g*, the fraction

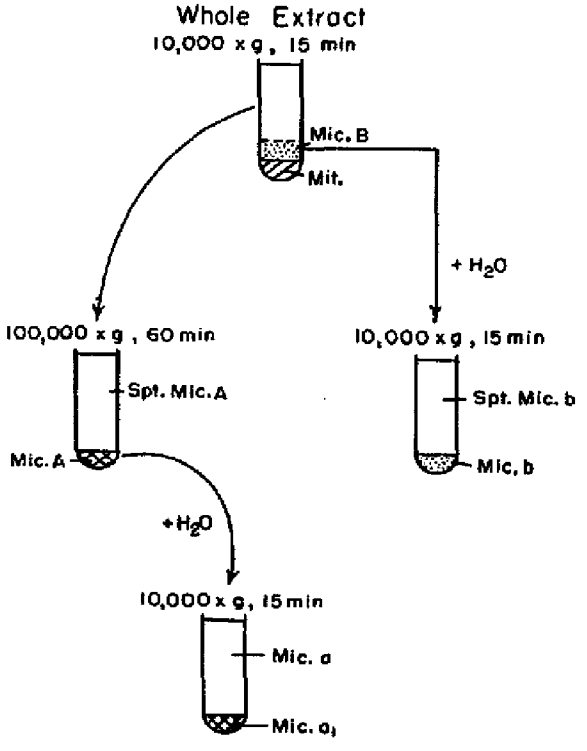

Fig. 1. Fractionation scheme for extracts from pea seedlings.

designated as "mitochondria" appeared as a tightly packed pellet. Above it always appeared a semifluid, cloudy zone, here designated as "microsomes *B*"; this was often rather difficult to separate cleanly from the supernatant, so that in several of the experiments "microsomes *A*", obtained by centrifuging the supernatant at 100,000 × *g* for 60 min, are contaminated with some "microsomes *B*".

The different "microsome" fractions were taken up in distilled water and gently homogenized by hand in an all glass homogenizer. The suspension of "microsomes *A*" was never completely clear but could be clarified by recentrifuging the suspension at

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10,000 × *g* for 15 min. The clear supernatant was designated as "microsomes *a*" and the sediment as "microsomes a_1". Since the latter proved to be inactive, most experiments were carried out with whole A fraction. The *B* fraction was also taken up in distilled water and recentrifuged at 10,000 × *g* for 15 min. The sediment was designated as "microsomes *b*" and used in the experiments.

Aliquots of the *a* and *b* fractions were diluted with distilled water to an optical density at 260 mμ of approx. 0.4 and examined in the electron microscope by Dr. R. C. WILLIAMS, whose help is here gratefully acknowledged.

In order to test the amino-acid-incorporating activity of the microsome fractions 0.4 ml of the aqueous suspension (containing 1–2 mg of protein) was added to 0.6 ml of a medium containing 50 μmoles potassium phosphate buffer, pH 7.5, 10 μmoles $MgCl_2$ and 1 μmole of DL-[1-^{14}C]phenylalanine containing 30,000 counts/min. Varying amounts of ATP and/or chloramphenicol (CA) were also added to test the effect of these agents. The crystalline di-potassium salt of ATP obtained from Pabst was used.

The suspensions were incubated aerobically in a Dubnoff metabolic incubator at 37° for periods of time from 10 to 150 min. At the end of the incubation periods the beakers were placed in ice and two or three 0.1 ml aliquots were withdrawn for determination of protein; 0.8 ml of 10 % (w/v) TCA were then added to the remaining solution. Zero time controls were run for each series. In the preparation of time curves, duplicate flasks were usually run for every point.

The TCA precipitates were filtered onto glass filter paper discs in a Tracerlab E-8B Precipitation Apparatus, and washed with 30 ml 5 % TCA, followed by 30 ml of absolute ethanol, 30 ml of a 2:1 ether–alcohol mixture and finally by dry ether. The papers were stored overnight in a vacuum desiccator over P_2O_5 and paraffin oil, weighed and counted in a Tracerlab scintillation counter.

Frequently, the first 10 ml of the filtrate plus 5 % TCA washings were collected in a volumetric flask, and protein was determined on three aliquots of this solution. It was considered to contain essentially all of the TCA-soluble protein.

Washing of the precipitate with hot TCA resulted in slightly higher specific activities. Since it did not, however, alter the shape of the time curves, and did result in less reproducible results, this step was usually omitted.

An empirical saturation curve was determined and the parameters obtained from it were used to calculate the specific activity of all the samples (counts/min/mg protein). Infinite thickness corresponded to 4.5 mg of protein.

The RNA content of the different fractions was roughly estimated from the absorption at 260 mμ, using $\varepsilon_{1\,cm}^{1\%} = 270$, after a rough correction for scattering[7].

The protein content was determined by the method of LOWRY *et al.*[8] using a standard of crystalline bovine serum albumin (BSA) obtained from Armour.

RESULTS

The results of a typical fractionation experiment of whole extract are given in Table I. It is seen that, as expected, the amino-acid-incorporating activity is roughly parallel to the RNA content of the two types of particles tested. The mitochondrial fraction was not tested here, since its specific activity is reportedly low[9]. It is interesting to note the lack of amino-acid-incorporating activity of microsomes a_1. It seems likely that they represent denatured particles, and their presence, in variable amounts, might

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TABLE I

RESULTS OF A TYPICAL FRACTIONATION EXPERIMENT OF WHOLE EXTRACT FROM PEA SEEDLINGS

Fraction	*Protein* % of total*	*RNA µg/mg protein*	*Specific activity** counts min/mg protein*
Mitochondria	9.6	180	not tested
Microsomes *b*	2.2	240	57
Spt. Microsomes *b*	3.2		
Microsomes *A*	9.2	450	100
Spt. Microsomes *A*	78.0		
Total	101.2		
Microsomes *a*	42***	—	240
Microsomes a_1	58	—	0

* Protein concentration in whole extract was 5–10 mg/ml. Total yield of protein in whole extract was 0.6–0.7 g/100 g fresh tissue.

** Tested in the presence of 1 µmole ATP/ml. Incubation time: 60 min. All counts are corrected for zero time uptake.

*** Given in percent of protein in Microsomes *A*.

TABLE II

YIELDS OF MICROSOMAL FRACTION FROM PEA SEEDLINGS

Expt. No.	*Age of seedlings days*	*Yield of mic. A mg protein/ g fresh tissue*	*Specific activity* counts/min/mg protein*
8a	5	0.30	68.3
8b**	5	0.22	62.0
9	7	0.51	120.0
10	4	0.40	70.0
12	7	0.33	60.0
14	4	0.59	100.0

* Incubated for 60 min in medium containing 1 µmole ATP/ml.

** Extract stored for 24 h at 0°.

account for the variations in the specific activity of the whole microsomal fraction *A* that is always encountered. A few experiments, reproduced in Table II, illustrate typical variations in the yield of microsomes *A* and in their specific activities. There is no apparent correlation between the age of the seedlings and the yield of microsomal particles, or between the latter and the specific activity. Storage of the extract at 0° for 24 h did not alter substantially the specific activity. Storage of the isolated microsome fraction likewise did not affect their over-all amino-acid-incorporating capacity.

When examined in the electron microscope, the *a* fraction appeared as rather homogeneous particles, similar to those described by Ts'o *et al.*[2]; the *b* fraction showed particles of varying sizes, on the average larger than those of *a*, but also clumps and undefined aggregates.

In order to obtain active particles it was important to keep the pH of the extract above pH 7.1. If the pH was accidentally dropped, the amino-acid-incorporating activity was substantially decreased or even abolished.

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TABLE III

NET CHANGES IN PROTEIN CONCENTRATION ACCOMPANYING THE INCORPORATION OF AMINO ACID INTO PARTICULATE FRACTIONS FROM PEA SEEDLINGS

Expt. No.	Fraction	ATP added µm/ml	Specific activity		Changes in total protein	
			60 min counts/min/mg protein	150 min	60 min µg/mg protein	150 min
10	Microsomes A *	1.0	77	91**	142	248**
		0.1	66	90**	129	288**
		none	69		167	
14	Microsomes A	1.0	98	239	57	232
		1.0	102		57	
		none	89		85	
16	Microsomes A	1.0	23	167	65	212
18	Microsomes A	1.0	110	180	82	248
14	Microsomes a	1.0	246		100	
		none	237		80	
	Microsomes a_1	1.0	0		none	
	Microsomes b	1.0	57		— 100	
		none	124		— 40	

* Freshly prepared microsomes; all others were stored overnight before incubation.
** Incubated for 90 min.

A net increase in protein—as measured by the LOWRY method—was always observed with all the microsomes A fractions tested, but not with any other fraction; with microsomes b, a net loss in protein was observed. The results of some representative experiments are given in Table III.

It is seen that generally there is no significant difference in the activity of fresh

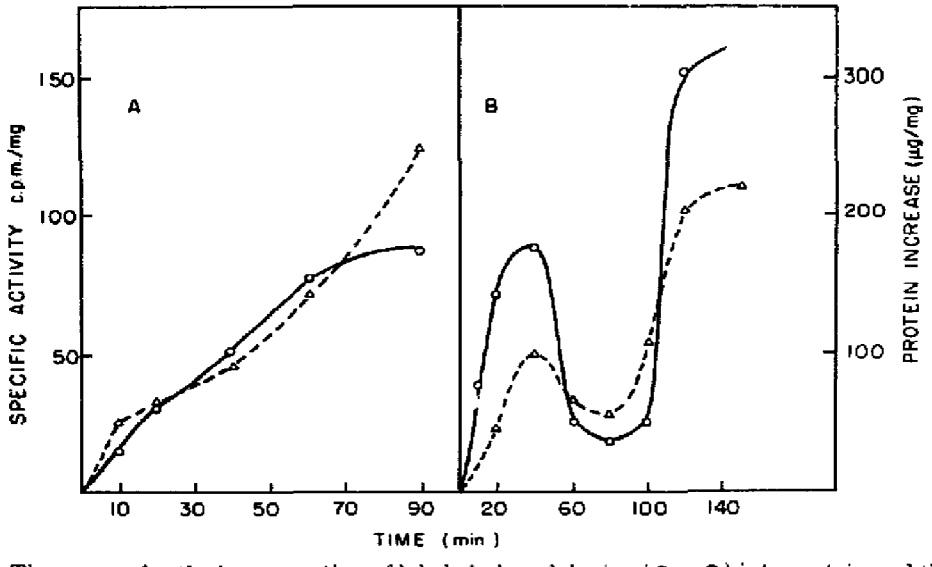

Fig. 2. Time curve for the incorporation of labeled phenylalanine (○—○) into protein and the net increase in protein (△---△) with microsomal particles. A: freshly prepared particles; B: particles stored overnight at 0°. One µmole ATP per ml added in both cases.

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and aged microsomal particles when the incorporation data at 60 min are compared. The effect of aging is, however, more clearly demonstrated by studying the incorporation of labeled amino acid and the increase in protein as a function of time. It is seen that whereas with fresh particles (Fig. 2A) protein increases at a more or less steady rate with a hardly noticeable break in the curve at 20 min, with aged particles (Fig. 2B) there is a sharp initial increase, followed by a decrease after 40 min, and again by a sharp rise in the radioactivity as well as in the total amount of protein after 90 min. The general shape of this curve is very characteristic of aged particles and was found to be reproducible, although the initial peak is not always as pronounced as that shown (see also Fig. 4). As a matter of fact, the peak was dismissed as experimental error in some early experiments of short duration, and the incorporation curves were drawn so as to resemble the typical saturation curves found for microsomal particles from liver[11,12], from ascites tumor[13] and reportedly also from peas[3].

Since in the present experiments the activity is measured on total protein precipitable by 5% TCA, it is not known whether the newly formed protein is secreted into the medium or is still associated with the particles.

It should be pointed out that the phosphate buffer is important for the activity of the particles. When "Tris" buffer of pH 7.6 was used for the preparation as well as for the incubation of the particles, the amino-acid-incorporating activity was substantially decreased (25 counts/min/mg as compared to 88 in phosphate, after 40 min) and the increase in protein was reduced to an insignificantly low level (20 μg as against 130 μg). Furthermore, the time curves for both activities followed rather straight lines and did not show the characteristic shape obtained in phosphate buffer.

The effect of added ATP on the incorporation of labeled amino acid was found to be variable and inconsistent. A concentration of 1 μmole/ml usually produced a slight stimulation (never more than 100%), but often it had no effect and sometimes it even was inhibitory. Washed microsomal particles were more susceptible to stimulation than were unwashed ones, as can be seen from Table IV.

TABLE IV

THE EFFECT OF ATP ON THE INCORPORATION OF LABELED AMINO ACID BY MICROSOMAL PARTICLES

Microsomes	*ATP added* μm/ml	*Specific activity* counts/min/mg protein*
Unwashed	none	118.3
	0.1	119.3
Washed	none	86.2
	0.1	101.0
	1.0	155.5

* Incubated for 60 min.

The effect of added ATP on the formation of protein often, although not always, seemed to be inhibitory (see Table III). As with the incorporation data, the results were inconsistent and rather nonsensical especially when data obtained after different incubation times were compared. These results became clearer, however, when time curves obtained with different amounts of added ATP were studied. It was found, as can be seen from Figs. 3A and 3B, that these curves cross each other at one or more

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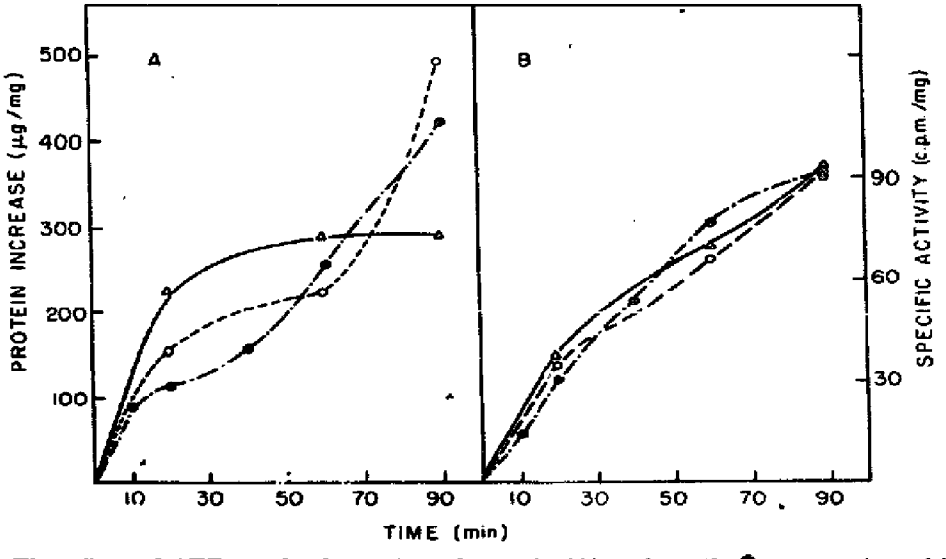

Fig. 3. The effect of ATP on the formation of protein (A) and on the incorporation of labeled phenylalanine (B) with freshly prepared microsomal particles. ●–·–● 1 μmole ATP; ○– – –○ 0.1 μmole ATP; △– – –△ no ATP added.

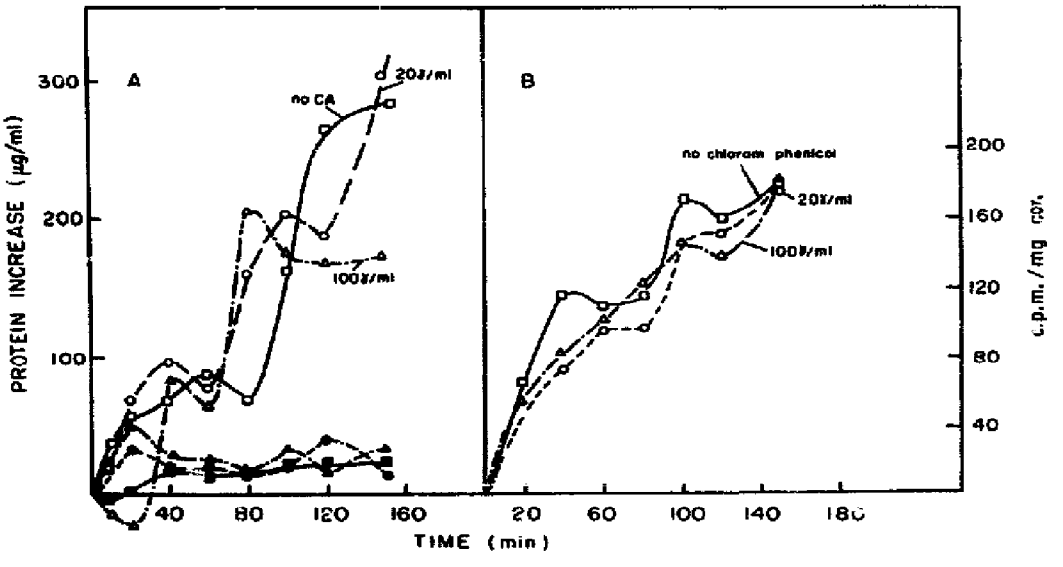

Fig. 4. The effect of chloramphenicol on the formation of protein (A) and on the incorporation of labeled phenylalanine (B) with microsomal particles *in vitro*. The particles were stored overnight before use, and 1 μmole ATP was added to each beaker. □—□ control; ○– –○ 20 μg CA per ml; △–·–△ 100 μg per ml. The open points designate protein precipitated by 5% TCA, whereas the solid ones designate TCA-soluble protein.

points, so that one may observe a stimulation or inhibition depending on the length of the incubation period.

After short times the apparent effect of added ATP is apt to be inhibitory, whereas after longer incubations one is more likely to observe a stimulation. However,

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since the effect of added ATP is superimposed on that of the ATP or its physiological equivalent already present in the particles, the exact shape of the curves will depend on the mode of preparation and age of the particles. Hence, no conclusions should be drawn from a single point on the time curve.

The present observations are helpful in interpreting some earlier somewhat conflicting reports by WEBSTER[3,6], on the effect of ATP on ribonucleoprotein particles from pea seedlings. It may be of interest that this author found inhibition of the incorporation of [^{14}C]glutamic acid by 1 μmole ATP when he used an incubation time of 30 min[6], but stimulation in another experimental series where longer incubation times were employed[3].

The effect of chloramphenicol (CA) has been tested in the present system since, although it is a well-known inhibitor of protein synthesis in whole cells, it has generally been found not to affect the incorporation of labeled amino acids by isolated microsomal particles from animal sources; but WEBSTER[14], working with pea particles, has reported an inhibition by high concentrations of CA. It was found that the over-all effect was indeed small, but that considerable differences could be observed at certain times. Typical time curves are illustrated by Figs. 4A and 4B. It seems that the effect is more pronounced on the total change in protein than on the incorporation of label. This might simply be due to the fact that the latter determinations are less precise than those of protein. The initial increase of TCA-soluble protein in the presence of CA (Fig. 4A) should be noted.

DISCUSSION

The uncertainity of whether the incorporation, *in vitro*, of labeled amino acids into the proteins of isolated microsomal particles does, in fact, represent a synthesis of protein or merely an exchange of amino acid residues has always haunted workers in this field; particularly because it has never been possible to demonstrate a net increase in the amount of protein[12,15] and because the addition of a mixture of amino acids does not seem to affect the incorporation of label[3,10,15,17,18]. Furthermore, it has not been possible, *in vitro*, to obtain the continuous labeling of the soluble protein—as opposed to particulate protein—which is so characteristic of labeling experiments *in vivo*.

The most direct evidence that the formation of new protein accompanies the incorporation of labeled amino acids *in vitro* was the demonstration by CAMPBELL, GREENGARD AND KERNOT[16] that the antigen–antibody complex isolated from rat liver microsomes after addition of rat albumin antiserum was at least five times more radioactive than the mixture of other microsomal proteins. Even in this case, however, a net increase in serum albumin or the formation of soluble protein could not be demonstrated.

In the present experiments it has now been demonstrated that under certain conditions the incorporation of labeled amino acids into proteins by isolated microsomal particles is indeed accompanied by a substantial net increase in the amount of protein. The general similarity between the curves for radioactivity and for protein (Fig. 2) are evidence that these two processes are closely related. It has, however, also been shown that a limited amount of labeling can be obtained without a concomitant net increase in protein (see Microsomes *b*, Table III). This, together with the particular shape of the incorporation and protein curves obtained with aged particles (see Figs. 2B and 4A)

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suggests that there is proteolytic as well as synthetic activity in these preparations. Experiments designed to elucidate the relationship, if any, between these two activities are underway.

Strictly speaking, the word "protein" should be substituted by "protein-like material", since no protein was actually isolated and characterized in the present experiments. The material which appears in the course of the incubation of the ribonucleoprotein particles is, however, proteinaceous, since: (1) it gives a positive test with the LOWRY reagent, which does not react with amino acids or small peptides; (2) the bulk of the material is precipitable by 5% TCA, as can be seen by the very small increase in TCA-soluble as compared to total "protein" (see Fig. 4A); (3) it has incorporated externally added labeled phenylalanine. The actual amount of protein formed is, of course, subject to correction as standards more appropriate than BSA will become available.

Since no amino acids, other than phenylalanine, were added, the system must contain the necessary precursors which it then transforms into LOWRY-positive, TCA-precipitable material. That this is a justified assumption has been shown by preliminary experiments with dialysed particles which show no activity when incubated with phenylalanine, Mg, and ATP, but synthesize protein in amounts comparable to undialysed ones when a mixture of 17 amino acids is added. These studies with dialysed microsomal particles are continuing.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professors A. D. MCLAREN and W. M. STANLEY, without whose generous support this investigation would have been impossible, to Professor D. M. GREENBERG for the use of a Spinco ultracentrifuge, and to Mr. T. C. BROYER for the use of the counting equipment.

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