[7] R. R. Race and R. Sanger, *Blood Groups in Man* (Oxford: Blackwell, Scientific Publications, 1954), p. 60.
[8] A. Gottschalk, *Physiol. Rev.*, **37**, 66, 1957.
[9] J. A. Morton and M. M. Pickles, *J. Clin. Pathol.*, **4**, 189, 1951.
[10] R. E. Rosenfield and P. Vogel, *Trans. N. Y. Acad. Sci.*, **13**, 213, 1951.
[11] W. M. Watkins and W. T. J. Morgan, *Brit. J. Exptl. Pathol.*, **35**, 181, 1954.
[12] C. Howe, J. D. MacLennan, I. Mandl, and E. A. Kabat, *J. Bacteriol.*, **74**, 365, 1957.

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*AMINO ACID INCORPORATION IN PIGEON PANCREAS FRACTIONS**

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The function of the pancreas is predominantly that of synthesis of proteins. This tissue has therefore attracted the attention of various workers interested in this problem, for example, Mirsky,[1] Hokin,[2] Straub.[3] However, those who have attempted to obtain cell-free preparations, either for protein synthesis proper or even for an amino acid incorporation, have encountered difficulties. Yet, as a protein-synthesizing system, pancreas holds considerable promise, and for this reason we embarked some years ago on a plan to explore, if possible, the path of protein synthesis in preparations obtained from this organ.[4, 5]

At first, we also encountered difficulties in getting not only net synthesis but also amino acid incorporation, and as a preliminary we concentrated on the characterization of amino acid-activating enzymes of the type first demonstrated by Hoagland in the liver,[6] which we found rather concentrated in pancreas extracts. The trytophan-activating enzyme was particularly abundant in beef pancreas, and a fraction was obtained from particle-free extracts of this organ representing 70 per cent pure enzyme protein.[4] Studies with this enzyme have given more detailed information on the character and mechanism of action of this type of enzyme.[5]

Meanwhile, although unable to obtain in vitro enzyme synthesis in cell-free pancreas preparations, we turned, as a more hopeful approach, to amino acid incorporation. After considerable trial and error, it was found that the pancreas of young pigeons, 6–8 weeks of age, gave homogenates that consistently incorporated amino acid into protein. At first, the pancreas system appeared to be quite similar to the liver system of Zamecnik and Keller;[7] by recombining the supernate and microsome fractions, incorporation was obtained, while microsomes and supernate alone showed only slight activity. It then appeared, however, that the supernate fraction behaved differently in the pancreas from that of the liver, since reactivation could be obtained with heated supernate, while, in the liver, heating of the supernate destroyed activity. The heat-stable factor in the pancreas was then identified as a compound similar to the polynucleotide fraction of liver recently described by Hoagland, Zamecnik, and Stephenson.[8] The pancreas system therefore appeared suited to characterize this component, and it is on attempts to do this that we want to report here.

Fractionation of Pancreas Homogenate.—The microsomal fraction was prepared from the pancreases of 6–8-week-old pigeons by a procedure similar to that described for liver by Zamecnik and Keller,[7] for guinea-pig pancreas by Palade,[9] and for dog pancreas by Hokin.[10] The pancreas was quickly removed from pigeons killed by decapitation and homogenized in 5 volumes of ice-cold 15 per cent sucrose, in a glass homogenizer of the Bucher type.[11] Lower concentrations of sucrose give less effective fractionation, while higher concentrations of sucrose give less active particles. Nuclei, debris, and whole cells were removed by filtering through fine gauze and by centrifugation for 5 minutes at 500 *g* at 0°. The zymogen granules were removed by centrifugation at 2000 *g* for 15 minutes. At this point, three more volumes of cold 15 per cent sucrose were added, and the mitochondrial fraction was centrifuged off at 9000 *g* for 15 minutes at 0°, the high-speed head of an International centrifuge being used. The microsomal fraction was then obtained by centrifugation at 105,000 *g* for 1 hour at 0°, with the preparative Spinco Model L, and was resuspended in 8.5 per cent sucrose to a concentration of approximately 25 mg. of protein/ml. The clear 105,000 *g* supernatant or derivative preparations became the main object of our studies.

Estimation of Protein-incorporated Radioactivity.—After incubation, the reaction was stopped by the addition of 5 ml. of a 5 per cent solution of trichloracetic acid.[12] The precipitate was collected by centrifugation and washed three times with a similar volume of cold TCA. The residue was washed three times with 3:1 ethanol-ether and then heated at 90° for 15 minutes in 5 per cent TCA. The TCA was removed, and the residue washed once more with ethanol-ether. The white precipitate was transferred to nickel dishes, dried, and counted in a windowless gas-flow counter (Nuclear Model C-110A). The weight of each sample was accurately determined. This value was used for self-absorption corrections. The effectiveness of this washing procedure was tested by controls in which the TCA was added prior to the enzyme.

Procedures for the preparation of heated pigeon supernatant and phenol-extracted supernatant and for the counting of nucleotide-bound radioactive material are described in Figure 1 and Table 6.

The C^{14}-DL-amino acids used in this work were purchased from New England Nuclear Corporation. The authors wish to express their gratitude to Dr. Robert Loftfield, of the Huntington Memorial Laboratories at the Massachusetts General Hospital, for the preparation of L-leucine-1-C^{14}. Phosphoenolpyruvate was purchased from the California Foundation for Biochemical Research. Crystalline phosphoenolpyruvate kinase was prepared from rabbit muscle and kindly supplied by Dr. John Gregory. Lithium carbamyl phosphate and carbamyl phosphate kinase were prepared by Dr. M. E. Jones and Dr. H. Peck. A rather crude *Streptococcus faecalis* extract was free of interfering enzymes and could be used as CAP kinase. The nucleotides used in this work were the products of the Pabst Laboratories.

The Incorporation of C^{14} Leucine into Pigeon Pancreas Homogenate.—Initially we met difficulties in obtaining incorporation, and only when turning to pigeon pancreas did results become encouraging. Even with pigeon pancreas, only with the organs of 6–8 week-old animals were consistently reproducible activities obtained. The

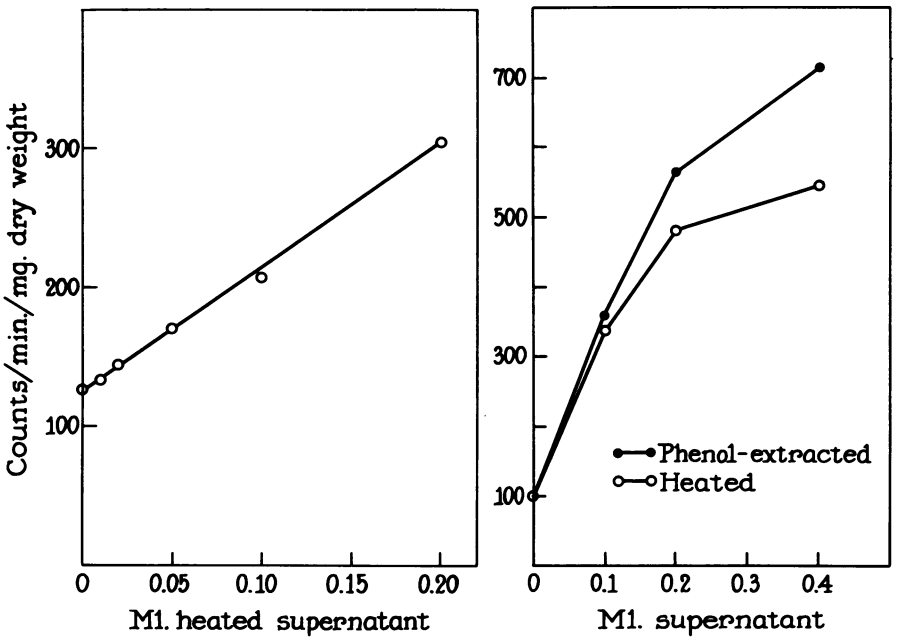

FIG. 1.—Effect of varied concentrations of heated and phenol extracted supernatant on incorporation.

Heated supernatant was prepared by placing 4 gm. of minced pigeon pancreas in 4 ml. of water, in a boiling bath for 15 minutes. A jet of hot steam was introduced to the mince at the onset of boiling, for 1 minute, to raise the temperature rapidly. The denatured preparation was cooled, the volume adjusted to 20 ml. with water, and then the tissue was homogenized with a tight-fitting glass homogenizer. Clear supernatant was obtained after spinning in the Spinco Model L at 75,000 *g* for 30 minutes. Phenol-extracted supernatant was prepared by mechanical mixing of heated supernatant with an equal volume of 90 per cent phenol (*w/v*) for 30 minutes at 0° (see n. 16). The water-phenol mixture was centrifuged at 20,000 *g* in the Servall and the water layer decanted from the phenol + precipitate. This extraction was repeated once more. The combined aqueous layers were lyophilized overnight, and the residue was suspended in cold water to half the volume of the original sample. Slight traces of phenol remained.

The heated supernatant was lyophilized and resuspended in half the original volume in water. The conditions for the enzyme incubation were identical with those of Table 1. Each tube contained approximately 4.2 mg. of microsomal protein.

first task now was to test activities in various fractions or combinations thereof. In Table 1, a survey of such experiments is presented. It shows that, as in liver,[7] incorporation into protein depends on a combination of microsome and supernatant fraction. It is shown in Tables 1 and 2 that, without an energy source, i.e., a phosphate bond feeder +ATP, no incorporation whatsoever is obtained. With supernatant alone, no incorporation into protein was seen, but microsomes retained about one-third to one-fourth of the maximum activity elicited on addition of supernatant. The addition of 0.2 μg. of crystalline pancreatic ribonuclease completely inhibited the activity of the combined fractions.

The highest incorporation of leucine took place in the pH range 8.0–8.2. The activity of the system fell off rapidly at pH values below 7.8 and above 8.4.

The enzymatic incorporation of leucine into microsomal protein required the presence of magnesium. Optimal incorporation occurred when the magnesium ion concentration was 0.005M. Manganese or calcium at equivalent concentrations were unable to activate the system.

For optimal activity, the system also required the presence of added salts. KCl was more effective than NaCl in stimulating leucine incorporation. At concentrations of NaCl higher than 0.05 M, the activity fell. At a concentration of 0.05 M, KCl gave optimal activity and remained constant up to 0.10 M, after which the activity dropped.

The system appeared to be saturated at 5×10^{-5} M L-leucine, and in 15 minutes 80 per cent of the incorporation had taken place.

TABLE 1*

INCORPORATION OF LEUCINE-1-C^{14} INTO PIGEON PANCREAS FRACTIONS

Fractions	Homogenate†	c.p.m./Mg. Dry Wt.
		472
Single	9,000 g (mitochondria)	75
	105,000 g (microsomes)	160
	Supernatant	20
Combined	Mitochondria + supernatant	241
	Microsomes + supernatant	593
	Microsomes + supernatant but without ATP and CAP	9

* Each tube contained 5 μmoles of $MgCl_2$; 50 μmoles of Tris buffer of pH 8.1; 50 μmoles of KCl; 0.1 μmoles of DL-leucine-C^{14} (7.5×10^6 c.p.m. per micromole); 10 μmoles CAP, CAP kinase (crude extract of *Streptococcus faecalis*); 1 μmole of ATP; 0.4 μmole of GTP; and supernatant derived from approximately 0.2 gm. wet weight of pigeon pancreas. The final volume of the system was 1.0 ml. The tubes were incubated for 30 minutes at 37°.

† After removal of cell debris, nuclei, and zymogen granules by 2,000 g centrifugation.

TABLE 2*

EFFECT OF VARIOUS SUPERNATANT FRACTIONS ON LEUCINE INCORPORATION INTO PIGEON PANCREAS MICROSOMES

Additions	c.p.m./Mg. Dry Wt.
None	182
Whole supernatant	488
pH 5 precipitate	147
pH 5 supernatant	325
Heated† whole supernatant	470
Heated whole supernatant but without ATP and CAP	10

* To the 100,000 × g supernatant (whole supernatant) from pigeon pancreas, molar acetic acid was added in small portions at 0° until pH 5 was reached. The pH 5 precipitate and supernatant were collected by centrifugation and neutralized with solid $KHCO_3$. The volume of the pH 5 precipitate was readjusted to the initial volume. The conditions for incubation were identical with those of Table 1. Each tube contained approximately 4.0 mg. of microsomal protein.

† Kept for 15 minutes in boiling water.

It soon appeared, however, that the similarity to the liver system was not complete. As elaborated by Keller and Zamecnik and their collaborators[13] and as already concluded earlier by Siekevitz in Zamecnik's laboratory,[14] the activity which complements the microsomes is precipitated at pH 5. This pH 5 fraction, on further analysis, was found by Hoagland[6] and by Hoagland, Keller, and Zamecnik[15] to contain the amino acid-activating enzymes. The data of Table 2 illustrate the differences between pancreas and liver. The pH 5 precipitate of pancreas supernate did not complement the microsomes, while the pH 5 supernate did. Furthermore, as shown in No. 5 in Table 2, activity could be restored to the microsomes with boiled supernatant, although the reaction mixture contained excess GTP, which

Keller and Zamecnik[13] had found to be a component of the liver system. This effect of the boiled supernate was obtained not only with leucine but, as may be seen in Table 3, with a number of other amino acids also, and therefore appears to be of a general nature. It is with the preliminary identification of this heat-stable component that we shall be dealing mostly from now on.

TABLE 3*

STIMULATION BY BOILED SUPERNATANT ON INCORPORATION OF VARIOUS LABELED AMINO ACIDS INTO MICROSOMAL PROTEIN

Experiment No.	Labeled Amino Acid	Boiled Supernatant	c.p.m./Mg. Dry Wt.†
1	DL-Leucine-1-C^{14}	−	98
		+	357
2	DL-Serine-3-C^{14}	−	120
		+	341
3	DL-Valine-1-C^{14}	−	91
		+	379
4	DL-Alanine-1-C^{14}	−	133
		+	221
5	DL-Phenylalanine-3-C^{14}	−	132
		+	158

* The conditions for incubation were identical with those shown in Table 1 except for the concentrations of amino acid, which were as follows: 0.1 μmole DL-leucine-1-C^{14} (7.5×10^6 c.p.m. per micromole); 0.4 μmole DL-serine-3-C^{14} (3.1×10^6 c.p.m. per micromole); 0.4 μmole DL-valine-1-C^{14} (2.0×10^6 c.p.m. per micromole); 0.4 μmole DL-alanine-1-C^{14} (3.7×10^6 c.p.m. per micromole); and DL-phenylalanine-3-C^{14} (2.3×10^6 c.p.m. per micromole). Each tube contained approximately 4.3 mg. of microsomal protein as determined by biuret reagent; 0.40 ml. of boiled supernatant was used as prepared from a 20 per cent pigeon pancreas homogenate.

† For better comparison, the specific activities of the other amino acids were adjusted to that of leucine.

As shown in Figure 1, the heated supernate factor can be assayed with the microsome fraction. This activity of heated supernate was, at first, rather puzzling. It was found that the pancreas microsomes retained amino acid-activating enzymes in reasonably large amounts when assayed by pyrophosphate exchange. Even after washing twice, a considerable pyrophosphate exchange was found with amino acid mixture or leucine. As shown in Tables 4 and 5, however, with dilution or washing, the activation by the heated supernate is lost, and now incorporation requires unheated supernate. In view of the persistence of amino acid-dependent ATP-pyrophosphate exchange in the washed microsomes, the enzymatic factor washed out most easily may be an enzyme functioning in another phase of amino acid transfer. We have not yet attempted to characterize this function more closely.

Preliminary Characterization of the Heat-stable Complement.—The material remaining soluble on heating is predominantly of a polynucleotide nature. Unfortunately, no good source for this material has been found so far, except the pancreas of young pigeons. Liver also contains this complement; however, it is closely associated there with protein and is obtained protein-free only after phenol treatment.[8]

If heating of the supernatant is carried out slowly, large losses of activity occur. Also, on dialyzing unheated supernate, almost all activity is lost, while, after heating, the complement proved to be non-dialyzable. Most likely, in these cases, the factor is enzymatically inactivated, presumably by ribonuclease. We also believe enzymatic inactivation to be the cause for the inactivity we found with extracts, of slaughterhouse-obtained pancreas.

As mentioned, the activator is non-dialyzable. At first, there were difficulties in showing the non-dialyzability because dialysis seemed to destroy activity. However, after the cellophane tubes had been washed with versene, inactivation disappeared, and after dialysis all the activity was found inside the cellophane bag. When subjected to electrophoresis on the Geon 426 resin (Goodrich), the active material migrates rather fast. The peak of activity, illustrated by Figure 2, largely coincides with the peak of absorption at 260. Ultracentrifugation of the peak fraction of Figure 2, kindly carried out by Miss Mary Peterson, of the Sloan Kettering Institute, showed a broad peak corresponding to molecular weights between 20,000 and 40,000 and indicated gross inhomogeneity.

TABLE 4*

INCORPORATION INTO MICROSOMES SEDIMENTED FROM DILUTED PANCREAS HOMOGENATES

HOMOGENATE DILUTION FROM WHICH MICROSOMES PREPARED	C.P.M./MG DRY WT.		
	No Addition	Supernatant	Heated Supernatant
Routine	144	352	246
4×	105	392	127
6×	42	437	78

* The routine microsome preparation as described is fourfold diluted with regard to the initial pancreas homogenate. The 4× and 6× dilutions of the routine fraction were prepared by the addition of appropriate volumes of cold 15 per cent sucrose just prior to the sedimentation of particles at 105,000 *g*. The conditions for the enzyme incubation were identical with those shown in Table 1. Microsomal protein ranged between 4.0 and 4.5 mg. per tube for the various preparations; 0.4 ml. of the heated and unheated supernatant was used as prepared from a 20 per cent pigeon pancreas homogenate.

TABLE 5*

LOSS OF INCORPORATION BY WASHING OF MICROSOMES

	C.P.M./MG. DRY WT.		
	No Addition	Supernatant	Heated Supernatant
Microsomes	57	231	116
Microsomes, washed	37	179	37

* Washed microsomes were prepared by resuspending the 105,000 *g* sediment in 2 volumes of 8.5 per cent sucrose and resedimentation in the Spinco at 105,000 *g*. The conditions for the enzyme incubation were identical with those shown in Table 1, except that each tube contained 15 μmoles of CAP. Microsomal protein for each tube ranged between 3.4 and 3.8 mg; 0.4 ml. of heated and unheated supernatant was used, as prepared from a 20 per cent pigeon pancreas homogenate.

So far, the experiments reported indicate that a polynucleotide acted as a mediator between the amino acid-activating enzyme and the microsome. If this were so, then in the absence of the microsomes the polynucleotide should be charged with amino acid. To test for this, a preparation of the supernate was used which, through dialysis, was depleted of carrier. To this supernatant, containing the activating enzymatic factors, a concentrated preparation of the polynucleotide was added which contained about ten to fifteen times as much polynucleotide as was ordinarily used in the previous experiments. Such an experiment is presented in Table 6.

Two methods for collecting the polynucleotide and washing were used. By acid ethanol precipitation,[18] the polynucleotide as well as protein was collected. It is significant that counts disappear when this precipitate is treated with hot TCA,[19] which indicates that the labeled leucine is not incorporated into protein, in which case hot TCA would not have removed the counts. On the other hand, the ex-

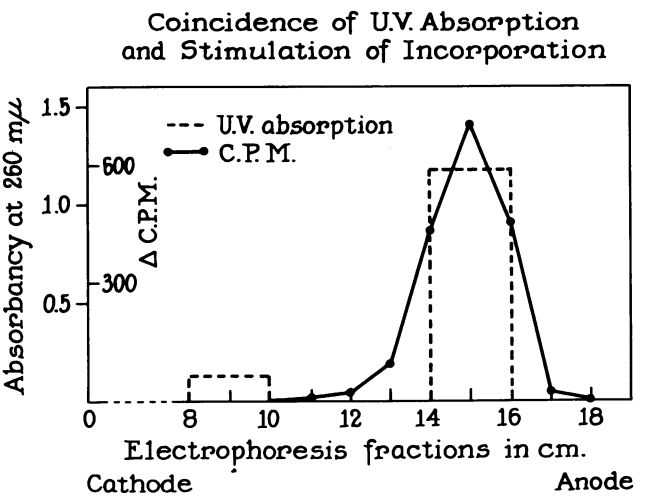

FIG. 2.—Coincidence of UV absorption and stimulation of incorporation. Concentrated phenol-extracted supernatant, prepared as described in Fig. 1, was dialyzed and electrophoresed on Geon 426 Resin for 19 hours at 150 volts in 0.1 molar tris buffer of pH 8.1 at 0°. After electrophoresis, the resin was cut into 1-cm. segments, and each fraction was eluted with water. Based on absorbancy at 260 millimicrons in the Beckman DU Spectrophotometer, 86 per cent of the material placed on the resin was recovered. Fractions 8–10 and 14–16 were combined and tested for activity toward the stimulation of incorporation into protein in the assay system. The conditions for the enzyme assay were identical to those of Table 1. The delta c.p.m. in this figure represent the total counts incorporated in the presence of the above combined fractions minus the counts incorporated by the microsomes alone.

TABLE 6*

TRANSFER OF CARBOXYL-LABELED LEUCINE TO POLYNUCLEOTIDE "FACTOR"

Incubation Mixture	A Washed Acid-Alcohol Ppt.	B Phenol-Extracted Non-dialyzable
	Total Counts/Min	
Boiled supernatant + "factor"	92	103
Supernatant, 0.25 ml. alone	49	43
Supernatant, 0.25 ml. + 0.4 ml. "factor" (= 5 ml. super.†)	2060	1993
Same	26 (hot TCA wash)	...

* Twenty per cent homogenate of pigeon pancreas in 15 per cent sucrose was centrifuged at 100,000 *g* for 1 hour in the Spinco Model L, and the clear supernatant was collected and dialyzed overnight against distilled water. Phenol-extracted supernatant concentrate, prepared as described in Fig. 1, was used as acceptor. 50 μmoles of tris buffer of pH 7.4; 5.0 μmoles of $MgCl_2$; 0.25 μmole of DL-leucine-1-C^{14}; 2.0 μmoles of ATP; 10 μmoles of CAP, CAP kinase; 0.25 ml. supernatant enzyme; and 0.40 ml. of factor (= 5 ml. supernatant) were incubated in a total volume of 1.0 ml. for 10 minutes at 37°. At the end of the reaction, phenol-extracted factor was added to those tubes which did not contain the material, and 2 mg. carrier yeast RNA was added to all vessels.

Set A: In one set of tubes, the reaction was stopped by the addition of a cold acid-ethanol reagent (935 ml. ethanol, 60 ml. glacial acetic acid, and 4.5 ml. of 2 *N* NaOH) and allowed to stand for 1 hour at 0° (see n. 17). The precipitate was centrifuged and washed five times with this reagent. The precipitate was then washed twice with cold 5 per cent TCA. Tube No. 4 was, in addition, extracted with TCA for 15 minutes at 90°. Finally, the residue was washed with ethanol-ether and counted for radioactivity.

Set B: In another set of tubes, 90 per cent phenol was added after incubation and extracted for 45 minutes at room temperature. The mixture was centrifuged at 10,000 *g*, and the clear top aqueous layer was removed by capillary pipette. The remaining phenol was extracted twice more with an equal volume of water. The aqueous portions were combined and subjected to exhaustive dialysis against water until no further counts could be detected in the dialyzate. Aliquots from each dialysis tubing were counted for radioactivity.

† Concentrate after phenol extraction.

traction of the same material with phenol, according to Kirby[16] and Gierer and Schramm,[17] more directly identified the counts as bound to the polynucleotide material of the same type as identified earlier with liver preparation by Hoagland *et al.*[8] as a factor in the amino acid incorporation. This experiment indicates that the polynucleotide accepts the activated amino acid in some manner.

Comments.—The experiments with cell-free preparations of pigeon pancreas amplify the experience of the Zamecnik-Hoagland group with liver preparation. It appears that, after the amino acid is activated, it is transferred to a polynucleotide carrier before it is eventually deposited in the microsome. This transfer presents a rather complex picture. The sequence of events is not quite clear yet, and a thorough discussion is better postponed until more details are available.

Summary.—In the study of cell-free preparations of pigeon pancreas, amino acid incorporation into microsome protein is shown to occur if a probably complex mixture of enzyme proteins and one or several polynucleotides is added. These polynucleotides are present in the non-particulate fraction of pigeon pancreas. They are heat-stable and non-dialyzable.

Amino acid transfer to the polynucleotide carrier can be shown on incubation with dialyzed supernate. It is concluded that this polynucleotide component acts as an intermediary between activating enzymes and microsomes, cf. also.[3]

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[1] V. Allfrey, M. M. Daly, and A. E. Mirsky, *J. Gen. Physiol.*, **37**, 157, 1953.

[2] L. E. Hokin, *Biochem. J.*, **48**, 320, 1951.

[3] F. B. Straub and A. Ullman, *Biochim. et Biophys. Acta*, **23**, 665, 1957.

[4] E. W. Davie, V. V. Koningsberger, and F. Lipmann, *Arch. Biochem. and Biophys.*, **65**, 21, 1956.

[5] N. Sharon and F. Lipmann, *Arch. Biochem. and Biophys.*, **69**, 219, 1957.

[6] M. B. Hoagland, *Biochim. et Biophys. Acta*, **16**, 288, 1955.

[7] P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.*, **209**, 337, 1954.

[8] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *Biochim. et Biophys. Acta*, **24**, 215, 1957.

[9] G. E. Palade and P. Siekevitz, *J. Biophys. Biochem. Cytol.*, **2**, 671, 1956.

[10] L. E. Hokin, *Biochim. et Biophys. Acta*, **18**, 379, 1955.

[11] N. L. R. Bucher, *J. Am. Chem. Soc.*, **75**, 498, 1953.

[12] The following abbreviations have been used: TCA, trichloracetic acid; PEP, phosphoenolpyruvate; CAP, carbamyl phosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; Tris, tris (hydroxymethyl) amino methane; c.p.m., counts per minutes.

[13] E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, **221**, 45, 1956.

[14] P. Siekevitz, *J. Biol. Chem.*, **195**, 549, 1952.

[15] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345, 1956.

[16] K. S. Kirby, *Biochem. J.*, **64**, 405, 1956.

[17] A. Gierer and G. Schramm, *Nature*, **177**, 702, 1956.

[18] R. Schucher and L. E. Hokin, *J. Biol. Chem.*, **210**, 551, 1954.

[19] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.*, **161**, 83, 1945.

[20] H. G. Kunkel, in D. Glick, *Methods of Biochemical Analysis* (New York, 1954), **1**, 141.