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The Effect of X-Irradiation on the Activation of Amino Acids in Nuclei from Rat Thymus Gland

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The activation of amino acids can be detected and measured by two methods (Hoagland, 1955). One measures the exchange of radioactive pyrophosphate into ATP in the presence of amino acids, and the other relies on the reaction of activated amino acyl adenylates with hydroxylamine to form amino acyl hydroxamates. These methods have given conflicting results when used by various authors to measure the activating enzymes in the same system (for collected references see Simkin, 1959; Novelli, 1960). With activating enzymes extracted from calf-thymus nuclei, Hopkins (1959) demonstrated pyrophosphate-ATP exchange, whereas Samarina, Zbarskii & Perevoshchikova (1960) found no hydroxamic acid formation. Failure to demonstrate activation on the addition of amino acids may be due to saturation of enzyme with high concentrations of endogenous amino acids rather than to an absence of the enzymes in question (Davie, Koningsberger & Lipmann, 1956). Purification procedures such as dialysis and absorption remove endogenous substrates but may also cause destruction of the enzyme (Clark, 1958; Allen, Glassman & Schweet, 1960). These difficulties now seem to have been resolved by hydroxamic acid formation which can detect activation of amino acids in the presence of endogenous substrates (Elliott & Coleman, 1962; Loftfield & Eigner, 1959).

The presence of phosphokinases (Osawa, Allfrey & Mirsky, 1957) and phosphatases (Creasey, 1960) in thymus nuclei is an added complication and provides a further argument in favour of the hydroxamate technique. Loftfield & Eigner (1959) separated the hydroxamic acids of neutral amino acids by means of ion-exchange paper chromatography, but by introducing an ionophoretic separation for the hydroxamates we were able to study the

activation of acidic, basic and neutral amino acids. By using isotopically labelled compounds we were able to observe the effect of X-irradiation on the activating enzymes both before and after extraction from the thymus nuclei.

METHODS AND MATERIALS

Animals. Animals weighing between 100 and 130 g. were taken from the Laboratory stock of Wistar rats. In most experiments thymus glands from female rats were used and no sex difference was observed.

Isolation of nuclei. Nuclear preparations were made by following the method of Creasey & Stocken (1959); medium A [sucrose (0.25 M), CaCl_2 (3.3 mM) and tris (5 mM), adjusted to pH 7.2 with N-HCl] was used generally, but their ionic medium C [glucose (45 mM), KCl (85 mM), NaCl (8.5 mM), CaCl_2 (2.5 mM), MgCl_2 (2.5 mM) and tris (5 mM), adjusted to pH 7.2 with N-HCl] was also tried. The presence of triethanolamine buffer in the original media interfered with the protein determination and was replaced with tris buffer. The nuclei were washed twice in each case with the isolation medium.

Preparation of amino acid-activating enzymes. The soluble activating enzymes were extracted from the nuclear pellet with ionic medium C from which glucose was omitted. For this extraction 20 strokes were made in an homogenizer of the Philpot & Stanier (1956) pattern. The difference in diameter of the tube and plunger was 0.003 in. The nuclei were removed by centrifuging (5 min. at 10 000 g). The pH 5.2 enzymes were prepared from the supernatant according to the procedure of Hoagland, Keller & Zamecnik (1956). The precipitated pH 5.2 enzymes were spun off (5 min. at 10 000 g) and immediately dissolved by homogenization in 0.1 M-tris-HCl buffer, pH 7.3. All the above operations were carried out at 0–2°.

Incubation mixture for amino acid activation. The final concentrations of reactants in the incubation medium were: ATP (disodium salt), 10 mM; MgCl_2 , 12 mM; hydroxylamine (low salt content, pH 7.3), 1 M, all in 50 mM-tris-HCl buffer, pH 7.3; and activating enzymes, 100 $\mu\text{g./ml.}$

Spectrophotometric assay of amino acid activation. The incubation mixture was 1 ml with respect to each of twelve L-amino acids alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, tryptophan and tyrosine. The pH of the amino acid mixture was adjusted to pH 7.3 and the final incubation volume was 5.5 ml. All incubations were carried out with gentle shaking at 37°. At appropriate intervals, 0, 30, 60, 90 and 120 min., samples (1.0 ml.) were taken and the hydroxamic acid contents measured as described by Schweet (1955). As internal standard an equimolar mixture of eight hydroxamates of alanine, arginine, glycine, isoleucine, leucine, phenylalanine, tyrosine and valine was used.

Isotopic assays. Labelled amino acids were activated separately, 1 μ C of an L-amino acid or 2 μ C of a DL-amino acid being included in the incubation mixture: the amounts were 454 μ m-moles of DL-[1-¹⁴C]alanine, 34.5 μ m-moles of L-[U-¹⁴C]arginine, 111.5 μ m-moles of L-[G-¹⁴C]glutamic acid, 259 μ m-moles of [2-¹⁴C]glycine, 3.44 μ m-moles of DL-[4,5-³H]leucine, 166 μ m-moles of L-[U-¹⁴C]lysine, 97 μ m-moles of L-[U-¹⁴C]phenylalanine and 39.4 μ m-moles of L-[U-¹⁴C]valine ('[U-¹⁴C]' indicates 'uniformly labelled'). A mixture of neutral L-amino acids (1 μ C) was included in the incubation medium when the effects of X-irradiation and of changes in pH on amino acid-activating enzymes were studied. The pH was 7.3 (unless stated otherwise) and the final incubation volume was 1.0 ml. At appropriate intervals samples (0.25 ml.) were taken, heated for 2 min. in a boiling-water bath and the denatured proteins centrifuged off (5 min. at 5000 g). The labelled amino acyl hydroxamic acids and the parent radioactive amino acid which are present in the clear supernatant were separated by two methods.

Activation of neutral amino acids was measured by the method of Loftfield & Eigner (1959). Samples (0.1 ml.) of the supernatant were evaporated on to a line 5 cm. from the end of a strip (2 cm. $\times$ 30 cm.) of Amberlite SA-2 cation-exchange paper (Na⁺ form). The chromatograms were developed as described by Loftfield & Eigner (1959) and dried. A 2.5 cm. band including the origin and shown to contain all the radioactive amino hydroxamic acids was removed from each chromatogram and assayed for radioactivity.

Activation of neutral as well as acidic and basic amino acids was measured by an electrophoretic method. Samples (0.1 ml.) of the supernatant were evaporated on to a line 28.5 cm. from the end of a strip (2 cm. $\times$ 57 cm.) of Whatman no. CM 50 cation-exchange paper. Electrophoresis was carried out at 35 v/cm. for 1 hr. in a Locarte high-voltage paper-electrophoresis apparatus; the buffer was pyridine-acetate (26.7 ml. of pyridine and 10 ml. of acetic acid/l.), pH 5.3. The ionograms were dried and sprayed with 5% (w/v) FeCl₃ in ethanol containing HCl (0.1 N) to detect amino hydroxamic acids or with 0.2% (w/v) ninhydrin in acetone to indicate amino acids. The strips were dried and scanned for radioactivity in an I.D.L. chromatogram scanner (model 2029) which was used in conjunction with a synchronously-driven chart recorder. Two radioactive peaks were observed on each ionogram which coincided with the movements of the free amino acid and the hydroxamate markers. A 2.5 cm. band that contained the radioactive hydroxamic acid was removed from the chromatogram and assayed for radioactivity.

Assay of radioactivity. Strips (2 cm. $\times$ 2.5 cm.) were

placed vertically in flat-bottom sample tubes (2 cm. wide $\times$ 5 cm. high) containing scintillation fluid (10 ml.), 0.4% (w/v) *p*-terphenyl and 0.04% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene-1,4-dioxan (7:3, v/v). The sample tube was arranged in an I.D.L. coincidence-scintillation counter (model 6012) so that maximum surface area of the inserted strip was presented to the two photomultiplier tubes. The efficiencies of the counter for ¹⁴C and ³H were 34 and 9% respectively. Sufficient counts were recorded to give a statistical error of less than 2%.

Adenosine triphosphate-generating system. Sodium phosphocreatine was prepared according to the method of Ennor & Stocken (1948). Creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) was isolated from rabbit muscle by 'procedure B' of Kuby, Noda & Lardy (1954). When the effect of ATP generation on amino acid activation was studied final concentrations of 50 mM-sodium phosphocreatine and 20 μ g. of creatine kinase/ml. were employed.

Yeast pyrophosphatase. This was prepared by the method of Bailey & Webb (1944) as modified by Rowles & Stocken (1950). The resultant solution (0.1 ml.) liberated 206 μ m-moles of orthophosphate P/mg. of protein/min. from Na₄P₂O₇ at 37° as determined by the method of Heppel (1955). In experiments to determine the effect of the enzyme on amino acid activation, it was present at a concentration of 20 μ g./ml.

Nuclear pyrophosphatase. A portion (3.0 ml.) of the activating enzymes in 0.1 M-tris-HCl buffer, pH 7.3, was used. The method described by Heppel (1955) was followed to determine the specific activity of this enzyme.

Determination of amino acids. The ninhydrin assay described by Yemm & Cocking (1955) was followed.

Determination of protein. This was done by the Folin-phenol method of Lowry, Rosebrough, Farr & Randall (1951). The standard was tyrosine. A factor of 4.22 was used to convert the results into protein equivalents. This factor was obtained from the colour given by a protein mixture isolated from nuclei of rat thymus gland.

Determination of nucleic acids. DNA and RNA were determined by the methods of Burton (1956) and Dische & Schwartz (1937) respectively.

Determination of inorganic phosphate. This was done by the method of Berenblum & Chain (1938).

Hydroxylamine with a low salt content. This was prepared by the method of Beinert *et al.* (1953), and the concentration of the hydroxylamine solution was determined spectrophotometrically (Frear & Burrell, 1955).

Synthesis of amino hydroxamic acids. The ethyl esters of alanine, arginine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan, tyrosine and valine were prepared by the methods of Hori & Tsuchiya (1958) or Cunningham, Newbold, Spring & Stark (1949), and condensed with hydroxylamine (Safir & Williams, 1952). α -Glutamyl hydroxamate was prepared by the method of Wieland & Fritz (1953). The hydroxamic acids were twice recrystallized from aqueous ethanol after which each moved as a single spot on Whatman no. 1 with butan-1-ol-acetic acid-water (4:1:1, by vol.) as solvent (Coleman & Elliott, 1960).

Labelled amino acids. These were purchased from The Radiochemical Centre, Amersham, Bucks. The neutral amino acids, present in a ¹⁴C-labelled algal-protein hydrolysate, were separated from the acidic and basic amino

acids by paper chromatography. A sample (0.2 ml.) of protein hydrolysate (25 $\mu\text{C}/85 \mu\text{g.}$) was applied to a strip (4 cm. $\times$ 30 cm.) of Amberlite SA-2 cation-exchange paper and developed in 0.05M-sodium phosphate buffer, pH 7.0, by the ascending technique. After drying, a band (R_f 0.4–0.9), which was shown to contain the neutral amino acids only, was removed from the chromatogram and eluted with water. The eluate was concentrated by freeze-drying and the specific radioactivity of the neutral-amino acid solution then determined.

X-irradiation. This was done at the Department of Radiotherapy, Churchill Hospital, Oxford. Characteristics of the Maximar X-ray machine were: half-value layer, 1.1 mm. of Cu; operating voltage, 215 kv at 15 ma; focal distance, 30 cm. Nuclear suspensions and activating enzymes extracted from the nuclei were irradiated at 0–2° in plastic cups of 15 ml. capacity. The dose rate was 165 r./min.

RESULTS

Determination of soluble protein in the supernatants obtained after preparation of the nuclei in sucrose showed that the ionic medium C extracted 4–5 times more protein from the nuclei than did the sucrose medium A. It was therefore decided to use iso-osmotic sucrose for the preparation of nuclei and ionic medium for the extraction of the activating enzymes. Wang (1961b) reported that 27% of the nuclear material of calf-thymus nuclei was extractable with 0.15M-sodium chloride.

The ratio of DNA P to RNA P in the intact nucleus was 15:1. Ionic medium extracted little DNA from the nucleus. The pH 5.2-enzyme fraction, obtained from the nuclear extract of six thymus glands, contained 480 $\mu\text{g.}$ of protein, 21 $\mu\text{g.}$ of RNA P and 1.32 $\mu\text{g.}$ of DNA P. Wang (1961a, b), on the basis of similar work, suggested that a number of proteins in addition to the metabolically active ribonucleoprotein could be extracted from nuclei. When the protein extract from nuclei was tested for activating enzyme a high activity was found (Table 1). Amino acyl hydroxamate formation proceeded linearly for about 60 min., after which it tapered off. When any of the reactants were omitted from the complete incubation medium, a decrease in amino acid activation resulted (Table 2).

Attempts were made to increase the rate of amino acid activation by the addition of an ATP-generating system or with yeast pyrophosphatase (Table 2). This met with only partial success because some pyrophosphatase is present in the pH 5.2 extract. The pyrophosphatase in the extract liberated 11.8 $\mu\text{m-moles}$ of orthophosphate P/mg. of protein/min. at 37° from tetrasodium pyrophosphate, whereas the added yeast pyrophosphatase preparation liberated 206 $\mu\text{m-moles}$ of orthophosphate P/mg. of protein/min. No free amino acids could be detected in the pH 5.2-activating-

enzyme preparation by the ninhydrin assay. Dialysis of the activating enzymes at 0° against six changes of water for 3 hr. had no detrimental effect on the activating ability. The addition of chloramphenicol (6.7 mm), an inhibitor of protein synthesis in liver nuclei (Rendi, 1959), to the incubation flasks had no effect on the activation of amino acids. This confirmed a finding of Hopkins (1959).

Because of the difference in ionic charge between an amino acid and its hydroxamic acid derivative an electrophoretic separation of the two compounds can conveniently be made. The ionophoretic mobilities of some amino acids and their corresponding hydroxamates on cation-exchange paper are recorded in Table 3. Migration on the negatively-charged cation-exchange paper was slower than on ordinary paper but had the advantage of a sharp separation. When they were studied by the isotopic hydroxamate method all the amino acids tested were activated (Table 4). To determine which were the more active activating enzymes in the nuclear extract, the hydroxamates, formed in an incubation medium that contained twelve amino acids, were separated by high-voltage paper electrophoresis. Ionophoretic separation was introduced in order to process greater quantities of material. Spots corresponding to alanyl, arginyl, leucyl and phenylalanyl hydroxamates were

Table 1. *Rate of formation of amino acyl hydroxamates assayed by spectrophotometry or radioactivity*

Nuclei were prepared from the thymus glands of six rats. Soluble proteins were extracted from the nuclei with ionic medium C. The activating enzymes were precipitated at pH 5.2, spun off (5 min. at 10 000 g) and immediately dissolved in 100 mM-tris-HCl buffer, pH 7.3. The incubation mixture for amino acid activation contained 1 vol. of activating-enzyme extract (200 $\mu\text{g.}$ of protein/ml.) and 1 vol. of a mixture containing ATP (20 mM), MgCl_2 (24 mM) and hydroxylamine (2M), pH 7.3. The incubation mixture was 1 mM with respect to each of twelve L-amino acids for the spectrophotometric assay or contained 1 μC of L-[^{14}C]valine for the isotopic assay. Incubation was carried out at 37° with shaking, and samples from the incubation medium were taken at intervals for determination of the hydroxamate content. Details of the assays are described in the text. The numbers in parentheses represent the numbers of experiments.

Incubation time (min.)	Amino acyl hydroxamates formed	
	Hydroxamate formed ($\mu\text{moles/mg.}$ of soluble protein)	Radioactivity incorporated into valyl hydroxamate (counts/min./ $\mu\text{g.}$ of soluble protein)
30	1.1 $\pm$ 0.04 (3)	36.2 $\pm$ 1.1 (3)
60	1.9 $\pm$ 0.07 (3)	49.5 $\pm$ 2.7 (3)
90	2.3 $\pm$ 0.07 (3)	61.7 $\pm$ 2.3 (3)
120	2.5 $\pm$ 0.08 (3)	67.8 $\pm$ 2.6 (3)

Table 2. *Effect of additions to and omissions from the incubation medium on the formation of hydroxamic acids*

Soluble proteins were extracted from nuclei with ionic medium C and the activating enzymes precipitated at pH 5.2. After centrifuging (5 min. at 10 000 g) the pellet of activating enzyme was dissolved in 100 mM-tris-HCl buffer, pH 7.3. The incubation mixture contained 1 vol. of activating-enzyme solution in 100 mM-tris-HCl buffer (200 μ g. of protein/ml.) and 1 vol. of an aqueous mixture containing ATP (20 mM), $MgCl_2$ (24 mM) and hydroxylamine (2M), pH 7.3. The incubation mixture was 1 mM with respect to each of twelve L-amino acids for the spectrophotometric assay or contained 1 μ C of L- $[^{14}C]$ valine for the isotopic assay. Yeast pyrophosphatase was added in a concentration of 20 μ g./ml. and the ATP-generating system contained sodium phosphocreatine (50 mM) and creatine kinase (20 μ g./ml.). Incubations were carried out at 37° for 1 hr. Details of the hydroxamate assays are described in the text. The numbers in parentheses indicate the numbers of experiments performed.

Changes in the complete system	Amino acyl hydroxamates formed	
	Hydroxamate formed (μ moles/mg. of soluble protein/hr.)	Radioactivity incorporated into valyl hydroxamate (counts/min./ μ g. of soluble protein/hr.)
Nil	1.97 $\pm$ 0.11 (3)	48.4 $\pm$ 3.8 (3)
ATP omitted	1.07 $\pm$ 0.14 (3)	24.2 $\pm$ 1.7 (3)
$MgCl_2$ omitted	1.55 $\pm$ 0.15 (3)	31.2 $\pm$ 4.9 (3)
NH_2OH omitted	0.12 $\pm$ 0.04 (3)	2.4 $\pm$ 0.4 (3)
Amino acids omitted	0.31 $\pm$ 0.01 (3)	—
Yeast pyrophosphatase added	2.02 $\pm$ 0.15 (3)	51.5 $\pm$ 2.6 (3)
ATP-generating system added	2.00 $\pm$ 0.07 (3)	49.3 $\pm$ 2.8 (3)

Table 3. *Electrophoretic mobilities of amino acids and amino acid hydroxamates on cation-exchange paper*

Solutions containing amino acids or amino hydroxamic acids were applied to strips (2 cm. $\times$ 57 cm.) of Whatman carboxymethylcellulose cation-exchange paper no. CM 50. Electrophoresis was carried out at 35 v/cm. for 1 hr. at 2°. The buffer was pyridine acetate (26.7 ml. of pyridine and 10 ml. of acetic acid/l.), pH 5.3. Each value is the average of two determinations.

Amino acid	$10^5 \times$ Electrophoretic mobility (cm. ² sec. ⁻¹ V ⁻¹)	
	Amino acid	Amino acid hydroxamate
Glu	-5.06	+3.03
Arg	+7.30	+3.93
His	+7.65	+3.93
Try	+2.13	+4.05
Lys	+8.09	+4.27
Tyr	+2.25	+4.50
Phe	+2.58	+6.07
Ileu	+2.58	+7.20
Leu	+2.58	+7.20
Ser	+2.02	+7.42
Val	+2.70	+7.64
Ala	+2.47	+8.55
Gly	+2.70	+9.10

detected. The optimum pH for the activation reaction was between 7.2 and 7.4 (Table 5). Other workers (Schweet & Allen, 1958; Davie *et al.* 1956) have found a pH optimum between 7.0 and 7.3 for the activating enzymes extracted from pancreas and studied by the hydroxamate method. Herranen

& Brunkhorst (1962) studied amino acid incorporation into nuclei isolated from thymus gland and found a pH optimum at 7.3.

Irradiation *in vitro* of the extracted activating enzymes with high doses (5000 r.) of X-rays failed to impair the activating properties of the enzymes. If, on the other hand, the nuclei were irradiated (in iso-osmotic sucrose medium) before the activating enzymes were extracted with ionic medium C, a marked decrease in enzymic activity was observed (Fig. 1). The soluble nuclear enzymes lost into the sucrose medium during the irradiation also exhibited this diminished activity (Table 6). After irradiation, the nuclei were centrifuged off (5 min. at 10000g) and the total protein in the sucrose solution was determined. These results are compared (Table 7) with the protein extractable from the nuclear pellet with ionic medium. Less protein was extractable in each case after the nuclei had been irradiated.

DISCUSSION

The significance of many of the findings in the present paper depends on the absence of appreciable cytoplasmic and whole-cell contamination. The most convenient guide to the purity of nuclei isolated from the thymus gland is a critical examination under the microscope. Preparations of nuclei were examined by phase-contrast microscopy and after staining. Very little cytoplasmic debris adhered to the nuclei, and whole-cell contamination was less than 3%. The ratio of

Table 4. *Activation of amino acids*

Amino acids labelled with ¹⁴ C or ³ H were activated separately; 1 μC of a L-amino acid or 2 μC of a DL-amino acid was included in the incubation mixture of 0.5 ml. of amino acid-activating enzymes (100 μg. of protein) in 100 mm-tris-HCl buffer, pH 7.3, and 0.5 ml. of a mixture containing ATP (20 mM), MgCl ₂ (24 mM) and hydroxylamine (2M), pH 7.3. The reaction was stopped after incubation for 1 hr. at 37°. The labelled amino acyl hydroxamic acids and the parent radioactive amino acid were separated on cation-exchange paper by electrophoresis, or by chromatography when only neutral amino acids were present. The radioactivity incorporated into the hydroxamate was then assayed in a coincidence scintillation counter. Details of the isotopic assay methods are described in the text. The numbers in parentheses indicate the numbers of individual assays.										
Amino acid tested	Ala	Arg	Glu	Gly	Leu	Lys	Phe	Val		
Radioactivity incorporated into amino acyl hydroxamates (counts/min./μg. of soluble protein/hr.)	27.7	57.5	21.2	19.5	11.0	39.4	38.6	48.4		
Amino acyl hydroxamates formed (μmoles/μg. of soluble protein/hr.)	8.350	2.620	3.120	6.680	0.095	8.660	4.960	2.520		
Concentration of L-amino acids added (μm-moles/μg. of soluble protein)	27.200	3.450	11.150	25.900	0.172	16.600	9.700	3.940		
10 ⁴ × Ratio of amino acyl hydroxamates formed/hr. to the concn. of L-amino acid added	3.7 (2)	7.6 (2)	2.8 (2)	2.6 (2)	5.5 (2)	5.2 (2)	5.1 (2)	6.4 (2)		

Table 5. *Effect of pH on the activation of amino acids*

[¹⁴C]Valine (1 μC) or a mixture of ¹⁴C-labelled neutral amino acids (1 μC) was added to an incubation mixture containing activating enzymes (100 μg. of protein), 50 μmoles of tris-HCl buffer, 10 μmoles of ATP, 12 μmoles of MgCl₂ and 1 m-mole of hydroxylamine. The pH of the mixture was adjusted with NaOH or HCl and the final volume was 1 ml. After incubation for 1 hr. at 37° the radioactive hydroxamates were separated from the parent amino acids by paper chromatography on Amberlite SA-2 cation-exchange paper (Na⁺ form) and assayed for radioactivity in a coincidence-scintillation counter. Details of the activation assays are described in the text.

Amino acyl hydroxamic acid formed (counts/min./μg. of soluble protein/hr.)		
pH	Valine	Neutral amino acid mixture
6.80	38.7	63.0
7.15	42.1	73.1
7.25	43.8	92.6
7.40	48.2	108.9
7.60	38.2	65.2
7.80	23.2	38.6
8.20	19.6	32.8

DNA P to RNA P of 15:1 is also an indication of the purity of nuclear preparations (Allfrey, Daly & Mirsky, 1955). The question of contamination by soluble cytoplasmic enzymes has been investigated by Hopkins (1959), who found no evidence of diffusion of soluble activating enzymes from the cytoplasm into the nucleus during the isolation of nuclei in iso-osmotic sucrose. Stern & Mirsky (1953) have shown that nuclei isolated in sucrose can

Fig. 1. Decrease in the amino acid-activating ability of enzymes extracted from nuclei that had been irradiated with X-rays. Nuclei isolated from rat thymus gland in sucrose medium A were irradiated with different doses of X-rays at 0–2°. After irradiation, the nuclei were centrifuged off and the soluble activating enzymes extracted from the nuclear pellet with ionic medium C. The activation of [¹⁴C]valine or a ¹⁴C-labelled neutral-amino acid mixture was followed by the isotopic method used for neutral amino acids. Experimental details are described in the Methods and Materials section.

Table 6. *Activation of amino acids in nuclei irradiated in sucrose medium*

Nuclei (25 mg.) were irradiated in sucrose medium (1.0 ml.), centrifuged (5 min. at 10 000 g) and the soluble amino acid-activating proteins extracted from the nuclear pellet with ionic medium C (1.0 ml.). The amino acid-activating ability of the soluble enzymes in the supernatant obtained by centrifuging for 5 min. at 10 000 g was then determined. Experimental details are described in the text.

Dose (r.)	Radioactivity in sucrose supernatant (counts/min./ μ g. of protein)	Total counts/min.	Radioactivity extracted by ionic medium from sedimented nuclei (counts/min./ μ g. of protein)	Total counts/min.
0	32.5	487	46.0	1748
1000	24.3	349	29.2	964
2000	21.0	294	26.2	827

Table 7. *Effect of X-irradiation on the extractability of soluble proteins from rat thymus nuclei*

Nuclei (25 mg.) were suspended in sucrose medium (1.0 ml.) for irradiation and then centrifuged off (5 min. at 10 000 g). Soluble proteins were extracted from the nuclear pellet with ionic medium C (1.0 ml.) and the nuclei removed by centrifuging (5 min. at 10 000 g). The total protein content in each supernatant was then determined. Experimental details are described in the Methods and Materials section.

Dose (r.)	Protein in sucrose supernatant (μ g. of protein/ μ g. of DNA P)	Protein extracted by ionic medium from sedimented nuclei (μ g. of protein/ μ g. of DNA P)
0	0.751	1.90
500	0.738	1.78
1000	0.719	1.65
2000	0.700	1.58

retain some of their water-soluble enzymes. It is believed that the retention of enzymes by thymus nuclei is related not only to the permeability properties of the nuclear membrane but also to the stability of the protein-nucleic acid bond. When nuclei were isolated in ionic medium, significant losses of soluble proteins from the nucleus were observed. It appears that the electrolytes in ionic medium may rupture the protein-nucleic acid bonds, with the result that some proteins are released which then diffuse through the nuclear membrane.

The soluble enzymes extracted from thymus nuclei activate L-amino acids in the presence of ATP and Mg^{2+} ions. The nucleus contains all the factors necessary for amino acid activation, i.e. activating enzymes, L-amino acids, Mg^{2+} ions, ATP, phosphorylating enzymes and pyrophosphatase. Activation of amino acids in the nucleus *in vivo* may well proceed according to the scheme proposed by Hoagland (1955).

Objections to the spectrophotometric assay for amino acid activation are that it is relatively non-

sensitive and lacks specificity because hydroxylamine reacts also with fatty acid esters (Hestrin, 1950), acyl amino acid amides (Johnston, Mycek & Fruton, 1950) and anhydrides of amino acids. These disadvantages do not apply to the isotopic methods, which are extremely sensitive and which will demonstrate unambiguously the presence of activating enzymes in enzyme preparations of very low activity. Both the chromatographic and electrophoretic isotopic assays can conveniently be used in routine determinations of amino acid-activating enzymes. The ionophoretic method has the advantage that it can be used to study the activation of any amino acid as opposed to the chromatographic method which is suitable only for neutral amino acids.

Many workers have observed a high endogenous pyrophosphate-ATP exchange in the absence of added amino acids (Schweet, Holley & Allen, 1957; Novelli, 1958), and measurement of amino acid-activation rates by the exchange assay usually gave higher results than does the hydroxamate method (de Moss & Novelli, 1955; Novelli, 1958; Schweet & Allen, 1958). This is probably caused by the presence of pyrophosphatases, phosphokinases or other phosphatases in the enzyme extracts, and some care is required in the interpretation of observations if the exchange assay is used to measure amino acid activation in these preparations. Fluoride has been used to inhibit the interfering enzymes (Hoagland, 1955), but this may have only partial success because fluoride, above a certain concentration, also inhibits the activating enzymes (Davis & Novelli, 1958). Other workers found little inhibition of endogenous pyrophosphate-ATP exchange after the addition of fluoride (Schweet *et al.* 1957), and they removed the interfering enzymes by adsorption on charcoal. The use of the hydroxamate technique is therefore recommended when the activation of amino acids is to be studied in tissues where phosphatases are known to be present.

Activating enzymes extracted from nuclei that had been irradiated *in vitro* showed a decrease in

activating ability, but X-irradiation of the isolated enzymes caused no damage. It is known that irradiation *in vitro* oxidizes thiol groups (Barron & Flood, 1950; Barron, Dickman, Muntz & Singer, 1949), and decreases the thiol content of nuclei from liver (Kedrova, Antokol'skaya & Rodionov, 1961) and thymus gland (Deakin, Ord & Stocken, 1963), but the precise role played by thiols in nuclear amino acid activation remains to be clarified. Another factor to be considered is the labilization of DNA-protein links during irradiation (Hagen, 1960). This, together with oxidation of thiol groups, might alter the conformation of the activating enzymes and decrease their extractability from the nucleus. The decrease in activating-enzyme activity caused by X-irradiation is of the same order as the inhibition by X-irradiation of amino acid incorporation into nuclear proteins (Logan, Errera & Ficq, 1959; J. A. Smit, unpublished work). This suggests that the radiation lesion of the activating enzymes may be the rate-limiting factor in the subsequent reactions leading to protein synthesis.

SUMMARY

1. Amino acid-activating enzymes were extracted with an ionic medium from nuclei prepared in iso-osmotic sucrose.

2. Pyrophosphatase activity was found in this enzyme preparation.

3. Amino acyl hydroxamate formation was measured spectrophotometrically and also by isotopic methods that have the advantage of greater sensitivity. An electrophoretic isotopic method was used to study the activation of acidic, basic and neutral amino acids.

4. Amino acyl hydroxamate formation is critically dependent on the concentration of amino acids present. The activation of eight amino acids was demonstrated.

5. Activating enzymes extracted from nuclei X-irradiated *in vitro* had a decreased activating ability, but irradiation of the isolated activating enzymes caused no inhibition.

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Fatty Acid Composition of the Serum Lipids of Pigs Given Different Amounts of Linoleic Acid

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Nunn & Smedley-MacLean (1938) were the first to show that in essential-fatty acid deficiency a trienoic acid accumulated in liver lipids. This has since been identified as an eicosatrienoic acid derived mainly from oleic acid (Mead & Slaton, 1956; Fulco & Mead, 1959).

During an investigation of the requirement of the pig for essential fatty acids, by using the trienoic acid:tetraenoic acid ratio as an index of deficiency (Holman, 1960), it was noted that although the lipids of serum, liver and heart had high contents of trienoic acid when diets low in linoleic acid were given, those of adipose tissue had very low amounts (Leat, 1962b). This suggested that as adipose tissue consists mainly of triglycerides the trienoic acid was associated with lipid fractions other than the triglycerides. That one of these fractions is probably phospholipid is indicated by the accumulation of high amounts of a trienoic acid in the serum phospholipids of dogs on low-fat diets (Wiese, Hansen & Baughman, 1957).

As large volumes of serum were available from pigs in various stages of essential-fatty acid deficiency it was considered of value to investigate further the distribution of eicosatrienoic acid and other fatty acids by more specific methods of separation and analysis. In this way changes in fatty acid pattern caused by gradual changes in dietary linoleate could be studied. A preliminary report has been published (Leat, 1962a).

EXPERIMENTAL

Animals. Six pigs were reared from 10 to 200 lb. live weight on diets containing respectively 0.07, 0.07, 0.30, 0.52, 0.97 and 3.67% of the calories as linoleic acid (Leat,

1962b). Olive oil provided the linoleate in the last four diets, replacing an isocaloric weight of carbohydrate (Table 1). At slaughter after overnight deprivation of food, the blood was collected, defibrinated and centrifuged to obtain the serum.

Extraction. A portion (100 ml.) of serum was poured, with stirring, into 500 ml. of ethanol-ether (3:1, v/v) and allowed to stand for 1 hr. in the dark with occasional shaking. The extract was filtered through a sintered-glass funnel and the protein precipitate extracted twice by a similar procedure using 200 ml. of solvent. Finally the protein cake was suspended in 100 ml. of methanol-chloroform (1:1, v/v) and allowed to stand overnight before filtering. The combined filtrates were evaporated almost to dryness at 40° in a rotary evaporator and the lipids dissolved in 5 ml. of methanol-chloroform (1:2, v/v).

Separation of lipids. The lipid extract was purified and separated into four fractions (as described by Böttcher, Woodford, Boelsma-van Houte & van Gent, 1959): (i) non-esterified fatty acids; (ii) sterol esters; (iii) triglycerides and free cholesterol; (iv) phospholipids. The fractions, with the exception of the phospholipids, were evaporated to dryness under a stream of nitrogen and were maintained in the dry state for the shortest possible time required to weigh them. They were then immediately redissolved in methanol-chloroform (1:2, v/v) and stored at 0° in the presence of quinol (5% of weight of lipid). The sterol ester and triglyceride fractions were saponified by refluxing for 30 min. with methanolic 0.5N-potassium hydroxide solution. The non-saponifiable fraction was removed by shaking twice with redistilled light petroleum (b.p. 40–60°). The fatty acids were liberated with N-sulphuric acid and extracted with light petroleum (3 × 20 ml.). The bulked light-petroleum extracts were then washed free of acid with water and dried over anhydrous sodium sulphate. The solvent was removed under a stream of nitrogen and the fatty acids were converted into their methyl esters by refluxing for 1 hr. with methanolic 2% (v/v) sulphuric acid. The methyl esters of the phospholipid fatty acids were obtained by refluxing for 3 hr. with methanolic 3N-hydrochloric acid. Quinol was added to all refluxing solutions to minimize oxidation.

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