Reaction of the Carcinogen Dimethylnitrosamine with Nucleic Acids *in vivo*

By VALDA M. CRADDOCK AND P. N. MAGEE

Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

(Received 20 March 1963)

Dimethylnitrosamine is a hepatotoxin and carcinogen whose administration is known to bring about a specific chemical alteration in the RNA of the liver cell. Magee & Farber (1962) showed that the administration of dimethylnitrosamine results in the alkylation of liver RNA specifically at N-7 of the guanine residues, and evidence was presented suggesting a similar reaction with liver DNA and with kidney RNA.

The present paper describes experiments on the metabolic fate of the methylated nucleic acids. Rats were injected with sufficient [^{14}C]dimethylnitrosamine to cause liver necrosis and induce kidney tumours in a small proportion of the animals. At various times the rats were killed, and the liver nucleic acids and kidney nucleic acids were isolated and assayed for radioactivity. Since dimethylnitrosamine is metabolized to form a normally occurring C_1 compound, which enters the C_1 metabolic pool, there is incorporation of radioactivity into normal bases of the nucleic acids as well as into 7-methylguanine. It was therefore necessary to separate the bases and to determine the radioactivity due to 7-methylguanine, in order to study the persistence of the ^{14}C -labelled methyl group in the nucleic acids *in vivo*.

A preliminary report of this work has appeared (Magee & Craddock, 1962).

MATERIALS AND METHODS

Dimethylnitrosamine. Material supplied by British Drug Houses Ltd. was redistilled before use. [^{14}C]Dimethylnitrosamine was prepared by Dr D. F. Heath by the method of Dutton & Heath (1956). The purity, checked by isotope dilution, was shown to be at least 99%.

Animals. Wistar albino rats of the Porton strain were maintained on MRC diet 41B (Bruce & Parkes, 1956). Female rats, about 200 g. body wt., were used.

Animal experiments. Groups of rats were given intraperitoneal injections of [^{14}C]dimethylnitrosamine, suitably diluted with carrier, in aq. 0.9% sodium chloride. The dose was 30 mg./kg. (10 μC /kg. in experiments on RNA, and 30 μC /kg. in experiments on DNA, except where otherwise stated). Usually five rats were used in experiments involving the isolation of RNA, and ten rats in those with DNA.

The animals were starved for 16 hr. before they were killed, to diminish liver glycogen. At the time-intervals stated, the animals were killed by a blow on the head, and the liver, kidney, pancreas and spleen were placed in liquid N_2 or on solid CO_2 as required. In experiments involving fractionation of subcellular components, liver was placed in 0.3 M-sucrose, and homogenized and fractionated by a slight modification of the method of Schneider & Hogeboom (1950).

Preparation of ribonucleic acid. RNA was prepared as described by Magee & Farber (1962).

Preparation of deoxyribonucleic acid. DNA was prepared by the *p*-aminosalicylate method of Kirby (1957), with omission of the dialysis procedure. Samples were tested for contamination with RNA by the method of Webb (1956), and for protein contamination by hydrolysis in 5 N-HCl at 110° for 16 hr., followed by paper-chromatographic examination in butan-1-ol-acetic acid-water (12:3:5, by vol.) for amino acids.

Hydrolysis and chromatography of nucleic acids. DNA and RNA samples were hydrolysed in 1 N-HCl at 100° for 1 hr., and the hydrolysates were subjected to ion-exchange chromatography as described by Magee & Farber (1962). The extinction of each fraction was measured at 260 m μ on a Unicam SP. 500 instrument. Paper chromatography was carried out in propan-2-ol-ammonia as described by Magee & Farber (1962). Radioactive peaks on paper were located with an automatic two-dimensional radiochromatogram scanner (Gauging Systems Ltd., Baldock, Herts.).

Histology. Samples of tissues were fixed by immersion in Helly's fluid or formol-calcium, embedded in paraffin, sectioned at 5 μ , and stained with haematoxylin and eosin.

Susceptibility of methylated ribonucleic acid to ribonuclease. Rat-liver RNA was treated with ribonuclease under the conditions of Kunitz (1940), and the extent of degradation was determined by measuring the extinction at 260 m μ of the filtrate obtained after the precipitation of unhydrolysed material with uranium acetate.

The nature of the breakdown products formed on treatment with ribonuclease was investigated by incubation of [^{14}C]methylated RNA in 0.1 M-sodium acetate buffer, pH 5, with ribonuclease at 40° for 2 hr., and dialysing the low-molecular-weight products in a rotating Visking tube against water with repeated changes of the diffusate (at 1½ hr., 1 hr. and 1½ hr. intervals). The diffusates were pooled and concentrated in a stream of air, and HCl was added to the concentrate, and to the contents of the dialysis tube, to bring the final concentration to 1 N. The solutions were hydrolysed at 100° for 1 hr., and the hydrolysates submitted to column chromatography on Dowex 50 as described by

Magee & Farber (1962). Control experiments were carried out with water in place of ribonuclease solutions.

Stability of methylated deoxyribonucleic acid. The liver DNA studied had been isolated from rats receiving [^{14}C]-dimethylnitrosamine 5 hr. previously, at which time labelling due to incorporation of C_1 metabolites was very low compared with that due to methylation. Samples were dialysed at room temperature, in 0.5 M-sodium acetate buffer, pH 5. High salt concentrations (1.5 M-ammonium acetate) were used in other experiments to free any breakdown products that might have been formed and that might have remained bound to undegraded DNA by electrostatic attraction. The diffusates and the solutions remaining in the dialysis tubes were evaporated, the ammonium acetate was removed by sublimation, and the residue was transferred to plastic planchets and assayed for radioactivity.

Radioactivity measurements. Dry solid RNA preparations were counted at 'infinite thickness' on aluminium planchets (area 1 cm^2). Fractions from column chromatography were reduced to a small volume by blowing down in a stream of air, transferred to plastic planchets (area 4 cm^2), dried, and counted at 'infinite thinness'. Measurements were made on an end-window counter with an automatic sample changer (Nuclear-Chicago Corp.), to an accuracy of 3% or more.

RESULTS

Methylation of ribonucleic acid

The radioactivity of RNA from liver, kidney, spleen and pancreas of rats given [^{14}C]-dimethylnitrosamine and killed after increasing intervals is shown in Fig. 1. This includes radioactivity due to methylation and also to incorporation of normal ^{14}C -labelled compounds of the 'active C_1 ' metabolic pool. The radioactivity of liver RNA at both doses of dimethylnitrosamine reaches a maximum within the first 12 hr. after injection and then decreases, at first rapidly and then more slowly. At 21 days the radioactivity has become very low (20 counts/min./

cm^2). The behaviour of the kidney RNA is different in that a 'plateau' of radioactivity is reached during the first 24 hr. and maintained for at least 7 days. At 21 days the radioactivity has fallen considerably, to 53 counts/min./ cm^2 , but is still higher than that of the liver RNA. The spleen, like the liver, shows maximum radioactivity during the first 24 hr., followed by a steady decline. The pancreas incorporates very little radioactivity at all.

An example of the results obtained from ion-exchange chromatography of acid hydrolysates of RNA is shown in Fig. 2. The degree of methylation of the nucleic acids is shown in Fig. 3. It is expressed as total radioactivity in the 7-methylguanine peak (counts/min.) divided by the total extinction (i.e. the sum of the extinction coefficients of the appropriate fractions) of the guanine peak from the same column. Similar results were obtained when adenine was made the standard. It was assumed from work by Magee & Farber (1962), and from the effect of nitrous acid treatment of the material from kidney RNA, that the well-defined peak of radioactivity emerging between adenine and guanine was 7-methylguanine. The labelled methyl group is lost rapidly from liver RNA, 82% of the maximum methylation reached at 5 hr. after injection having been lost at 2 days (Fig. 3). The methyl group is lost more slowly from kidney RNA, for which the corresponding value is 51%. The results for the rate of methylation are consistent with what is known about the rate of metabolism of dimethylnitrosamine (Heath, 1962).

Fig. 1. Radioactivities of RNA preparations from organs of rats injected with [^{14}C]-dimethylnitrosamine (30 mg./kg. body wt.) intraperitoneally and killed after increasing time-intervals. Experimental details are given in the text. $\circ$ — $\circ$, Liver; $\triangle$ — $\triangle$, spleen; $\bullet$ — $\bullet$, kidney; $\square$ — $\square$, pancreas. RNA from rats receiving 10 mg. of dimethylnitrosamine/kg. body wt. $\circ$ — $\circ$ — $\circ$, liver, results corrected for the higher specific radioactivity of the dimethylnitrosamine administered.

Fig. 2. Ion-exchange chromatography of acid hydrolysate of RNA from liver of rats treated with [^{14}C]-dimethylnitrosamine (30 mg./kg. body wt.) 24 hr. previously. A Dowex 50 (H^+ form) column (1 cm. $\times$ 10 cm.), with gradient elution from 1 N- to 4 N-HCl, was used. Experimental details are given in the text. $\bullet$, $E_{260\text{ m}\mu}^1$; $\circ$, radioactivity (counts/min.). G, Guanine; A, adenine; M, 7-methylguanine.

Labelling of guanine in RNA through incorporation of normal $^{14}\text{C}_1$ metabolic-pool intermediates in liver and in kidney is shown in Fig. 4. In each case specific radioactivity of guanine reaches a peak after approx. 24 hr., the value then remaining constant for at least 7 days. This slow rate of loss of radioactivity is consistent with a slow rate of turnover of RNA. The rapid labelling is presumably due to a low incorporation of highly labelled precursors. The radioactivity of material under the adenine peak from the column chromatography is due

Fig. 3. Time-course of methylation of RNA in rat liver and kidney at increasing time-intervals after the injection of dimethylnitrosamine. The results were obtained from the same RNA preparations as in Fig. 1 after ion-exchange chromatography. Experimental details are given in the text. The degree of methylation is expressed as radioactivity in 7-methylguanine (counts/min.) divided by the total extinction of guanine (see the text). ○—○, Liver; ●—●, kidney, both after dimethylnitrosamine (30 mg./kg. body wt.); ○---○, liver, after dimethylnitrosamine (10 mg./kg. body wt.), results corrected for the higher specific radioactivity of the dimethylnitrosamine administered.

Fig. 4. Specific radioactivities of 'adenine' (i.e. adenine plus a radioactive contaminant that may be a methylated adenine) and guanine of RNA of rat liver and kidney after the injection of ^{14}C dimethylnitrosamine (30 mg./kg. body wt.). Experimental details are given in the text. ●, Liver guanine; ○, liver 'adenine'; ▲, kidney guanine; △, kidney 'adenine'.

partly to incorporation of $^{14}\text{C}_1$ metabolic precursors into adenine, and also to a peak of radioactivity moving slightly before adenine, which may represent a methylated adenine.

A lower dose of dimethylnitrosamine (10 mg./kg.) was insufficient to cause overt liver necrosis, as confirmed by histological examination, but resulted in a measurable degree of methylation of liver RNA. As shown in Fig. 3, loss of the methyl group again took place at a greater rate during the first 19 hr. than at later time-intervals.

Intracellular localization of methylated ribonucleic acid. As there is evidence that *N*-demethylation of dimethylnitrosamine is catalysed by a microsomal enzyme (Magee & Vandekar, 1958; Brouwers & Emmelot, 1960), it might be expected that microsomal RNA, present at the site of production of the postulated alkylating intermediate, would become more highly methylated than RNA of other fractions of the cell. To investigate this, livers from rats killed 0.5 hr. after the administration of dimethylnitrosamine (30 mg./kg.; $20\mu\text{C/kg.}$) were homogenized and fractionated, and the RNA was isolated from each cell fraction. The products were hydrolysed and subjected to column chromatography. The results (Table 1) show the proportion of the total radioactivity of the liver RNA and the extent of methylation to be greatest for microsomal RNA.

Enzymic hydrolysis of ribonucleic acid. To investigate the susceptibility of methylated RNA to enzymic degradation, preparations were treated with ribonuclease *in vitro*. RNA isolated from livers of rats killed 5 hr. after the administration of dimethylnitrosamine was hydrolysed at approximately the same rate as was normal rat-liver RNA. On incubation with ribonuclease, ^{14}C methylated RNA gave diffusible products that yielded 7-

Table 1. Labelling of liver ribonucleic acid after the administration of ^{14}C dimethylnitrosamine

The ^{14}C dimethylnitrosamine (30 mg./kg.; $20\mu\text{C/kg.}$) was administered 0.5 hr. before the labelling of the liver RNA was determined. Experimental details are given in the text. The specific radioactivity of intact RNA is given by the total counts/min. in purines and pyrimidine nucleotides divided by the total extinctions (i.e. sums of the extinction coefficients of the appropriate fractions) of the corresponding peaks from the chromatography column. The degree of methylation is calculated as the counts/min. in 7-methylguanine divided by the extinction (at $260\mu\mu$) in guanine.

Fraction of cell	Specific radioactivity of intact RNA	Degree of methylation
Nuclei	5.30	17.3
Mitochondria	5.68	17.3
Microsomes	8.07	27.8
Supernatant	5.49	17.0

methylguanine after acid hydrolysis. Treatment with ribonuclease, dialysis, and column chromatography of the diffusate without acid hydrolysis showed that free 7-methylguanine had not been liberated. Very little diffusible material (2%) giving rise to 7-methylguanine on acid hydrolysis was found in the control experiment. It therefore appears that methylated RNA is hydrolysed by ribonuclease to give nucleotides containing 7-methylguanine.

Methylation of deoxyribonucleic acid

Work by Magee & Farber (1962) had suggested that the administration of dimethylnitrosamine leads to methylation of DNA at position 7 of the guanine residues. To study further the nature of the labelled material moving in the position of 7-methylguanine on column chromatography of acid-hydrolysed DNA, an experiment was carried out in which six rats were given injections of dimethylnitrosamine of higher specific radioactivity than usual (30 mg./kg.; 250 μ C/kg.) and killed after 5 hr., and the kidney DNA was isolated. This was added to a preparation of kidney DNA isolated from 20 rats that had received dimethylnitrosamine (50 mg./kg.) 5 hr. previously. The pooled samples, after being tested for RNA contamination, were hydrolysed and submitted to column chromatography. A peak of radioactivity moving in the position of 7-methylguanine was isolated (Fig. 5). This material

was rechromatographed, to separate it further from adenine and guanine, and was then subjected to paper chromatography. The radioactive material was located with an automatic radiochromatogram scanner, and was found to give a single spot, with an R_f identical with that of 7-methylguanine. The addition of standard 7-methylguanine followed by ion-exchange chromatography gave superimposable chromatographic profiles for radioactivity and ultraviolet-absorbing material.

The sequence of events during the labelling of DNA was investigated by isolating DNA from the liver and kidney of rats at various times after the administration of dimethylnitrosamine. Contamination with RNA was always less than 0.6%, generally 0.2%, and contamination with protein was insignificant. The results (Fig. 6) show the 7-methylguanine to be lost rapidly from liver DNA, and more slowly from kidney DNA. Adenine and guanine of liver DNA, and to a smaller extent of kidney DNA, became labelled by incorporation of normal C_1 metabolic-pool intermediates (Fig. 7).

Fig. 5. Ion-exchange chromatography of acid hydrolysate of DNA from kidneys of six rats treated with [14 C]dimethylnitrosamine (30 mg./kg. body wt.) and 20 rats treated with unlabelled dimethylnitrosamine (50 mg./kg. body wt.) 0.5 hr. previously. A Dowex 50 (H^+ form) column (1 cm. $\times$ 10 cm.), with gradient elution from 1N- to 4N-HCl, was used. Experimental details are given in the text. ●, $E_{260}^{1\text{cm}}$; ○, radioactivity (counts/min.).

Fig. 6. Time-course of methylation of DNA after the injection of [14 C]dimethylnitrosamine (30 mg./kg. body wt.). Experimental details are given in the text. The degree of methylation is expressed as radioactivity in 7-methylguanine (counts/min.) divided by the total extinction of guanine (see the text). ○, Liver; ●, kidney.

Fig. 7. Specific radioactivities of 'adenine' (i.e. adenine plus a radioactive contaminant that may be a methylated adenine) and guanine of DNA of rat liver and kidney after the injection of [14 C]dimethylnitrosamine (30 mg./kg. body wt.). Experimental details are given in the text. ●, Liver guanine; ○, liver 'adenine'; ▲, kidney guanine; △, kidney 'adenine'.

The specific radioactivity of the kidney purines does not increase after the first day, but that of the liver purines continues to rise, as would be expected in a tissue undergoing regeneration.

In view of reports on the instability of alkylated DNA (Brookes & Lawley, 1961; Lett, Parkins & Alexander, 1962) it was necessary to investigate the stability of methylated DNA under the conditions of its isolation. Preliminary results indicate that DNA as finally isolated does not break down to form diffusible nucleotides, or to split out the labelled group, under the conditions studied. After acid hydrolysis of the material remaining in the dialysis bag, the radioactive products were found to be readily diffusible.

DISCUSSION

The present experiments give good evidence for the rapid methylation of RNA of liver and of kidney, after a single administration of dimethylnitrosamine, the maximum extent of methylation being reached 5–12 hr. after the injection with the dose used. The methylated nucleic acids then disappear from the tissues, the rate of loss being particularly great in liver, especially during the first 48 hr. after administration. However, it is possible that a very small amount of methylated RNA may remain in the kidney or liver cells for an indefinite period, and this residue may have great significance with respect to carcinogenesis.

There are several ways in which methylated RNA could be lost from the liver cell. As dimethylnitrosamine causes centrilobular necrosis, methylated RNA might be lost because of the increased permeability and breakdown of the cell. However, methylated RNA disappeared at a greater rate during the first 48 hr. even after a dose of dimethylnitrosamine that did not result in extensive necrosis. In addition, there was no parallel extensive loss of total RNA. It may be that the RNA that becomes most highly methylated is degraded at a greater rate than is lightly methylated RNA, but the mechanism is not known.

With DNA, further evidence is presented for methylation at position 7 of guanine. A high percentage of the radioactivity found in intact DNA as isolated can be accounted for by material moving at the rate of 7-methylguanine on chromatography on Dowex 50. Results of experiments involving dialysis of lightly methylated DNA samples imply that the ^{14}C -labelled methyl group is not rapidly split out under the conditions studied. However, it remains possible that another less stable product is also formed, and that this is lost during the isolation procedure.

Methylated DNA disappears to a large extent from liver 2 days after a dose of dimethylnitrosamine, but loss from kidney takes place more

slowly. Probably methylated RNA is degraded to form nucleotides that are lost from the cell, leaving no abnormal residue, but with alkylated DNA there is evidence (Lawley, 1957) that breakdown *in vitro* could take place by splitting out of the alkylated base. A similar process might also occur *in vivo*. This would leave an abnormal DNA, which would be labile at the point of loss of the purine, and may undergo main-chain scission with the formation of two abnormal DNA fragments.

As expected from the fact that oxidative *N*-demethylation of dimethylnitrosamine takes place chiefly in the liver, the degree of methylation of RNA and DNA (and therefore probably of other cell components) was higher in the liver than in kidney. However, a single injection of dimethylnitrosamine, at the dose used, leads to development of tumours in kidney but not in liver. This does not necessarily imply that methylation is not a significant event in carcinogenesis. It may be that, in liver, the cells that become most highly methylated are killed, whereas kidney cells that are less extensively methylated are able to survive.

SUMMARY

1. The time-sequence of methylation of liver and kidney RNA and DNA after the administration of [^{14}C]dimethylnitrosamine has been investigated.
2. Methylation of liver RNA and kidney RNA reaches a maximum 5–12 hr. after the injection of dimethylnitrosamine, and then decreases, reaching a very low level at 3 weeks. In the liver, where methylation is most extensive, there is a rapid initial rate of loss of methyl group from RNA during the first 2 days, which is not dependent on the concomitant necrosis.
3. Liver DNA and kidney DNA become rapidly methylated; loss of the methyl group occurs rapidly from liver, more slowly from kidney.
4. Rapid incorporation of normal C_1 metabolic-pool intermediates takes place into RNA and DNA of liver and kidney; with liver DNA, incorporation continues after the first 24 hr., owing to regeneration of the tissue.

We thank Dr D. F. Heath for the [^{14}C]dimethylnitrosamine, Dr P. Brookes for the authentic 7-methylguanine, and Mr J. A. E. Jarvis for skilled technical assistance.

REFERENCES

- Brookes, P. & Lawley, P. D. (1961). *Biochem. J.* **80**, 496.
Brouwers, J. A. J. & Emmelot, P. (1960). *Exp. Cell Res.* **19**, 467.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.

- Dutton, A. & Heath, D. F. (1956). *J. chem. Soc.* p. 1892.
Heath, D. F. (1962). *Biochem. J.* **85**, 72.
Kirby, K. S. (1957). *Biochem. J.* **66**, 495.
Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
Lawley, P. D. (1957). *Proc. chem. Soc., Lond.*, p. 290.
Lett, J., Parkins, G. & Alexander, P. (1962). *Arch. Biochem. Biophys.* **97**, 80.
Magee, P. N. & Craddock, V. M. (1962). *Biochem. J.* **85**, 17P.
Magee, P. N. & Farber, E. (1962). *Biochem. J.* **83**, 114.
Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Webb, J. M. (1956). *J. biol. Chem.* **221**, 635.

Biochem. J. (1963) **89**, 37

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

BY J. A. SMIT AND L. A. STOCKEN

Department of Biochemistry, University of Oxford

(Received 2 April 1963)

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 $\mu\text{g./ml.}$