THE INCORPORATION OF ^{15}N, ^{35}S AND ^{14}C INTO NUCLEIC ACIDS AND PROTEINS OF RAT LIVER

by

R. M. S. SMELLIE*, W. M. McINDOE AND J. N. DAVIDSON

Department of Biochemistry, The University of Glasgow (Scotland)

In a recent paper we have discussed the incorporation of radioactive phosphorus (^{32}P) into the deoxyribonucleic acid (DNA) of rabbit and rat liver and into the individual ribonucleotides of the ribonucleic acid (RNA) of the nuclei and of the various cytoplasmic fractions (mitochondria, microsomes and cell sap) separated by the process of differential centrifugation[27]. It was shown that in normal adult rat liver ^{32}P was incorporated very slowly into DNA but very rapidly into all nucleotides of nuclear RNA. Incorporation into the nucleotides of cytoplasmic RNA was less extensive than for nuclear RNA and was greater in cell sap than in the cytoplasmic particles. In this paper these experiments have been extended by the use of ^{15}N-glycine, ^{14}C-formate and ^{35}S-methionine to label the proteins as well as the nucleic acids.

EXPERIMENTAL METHODS

Albino rats of approximately 200 g from the departmental colony were used throughout.

Sodium ^{14}C-formate and ^{35}S-methionine were obtained from the Radiochemical Centre, Amersham. The former was administered to 5 rats by intramuscular injection in one experiment in a dose of 80 μc/100 g body weight 4 h before killing and in another experiment in a dose of 100 μc/100 g body weight 16 h before killing. ^{35}S-methionine was given by intramuscular injection to 4 rats in a dose of 62.5 μc/100 g body weight 6 h, and again 4 h, before killing.

^{15}N-glycine was synthesised from ^{15}N-ammonium nitrate by the method of SCHOENHEIMER AND RATNER[24]. The final product contained 20.92 atom per cent excess ^{15}N. It was administered intraperitoneally in sterile isotonic saline solution to 5 rats in 3 doses of 50 mg each given 24, 22 and 20 h before killing.

The animals were killed by exsanguination under ether anaesthesia and the livers perfused with cold 0.9% (w/v) NaCl solution. The liver tissue after removal was chilled, finely minced with scissors and ground in 0.25 *M* sucrose in a POTTER-ELVEHJEM[23] homogeniser fitted with a perspex pestle.

From the suspension the nuclear fraction was removed by centrifugation. A portion of the supernatant fluid was used for the analysis of whole cytoplasm while the remainder was taken for the separation of cytoplasmic fractions according to the procedure previously described[27].

The mitochondria, microsomes and cell sap obtained in this way were extracted with 10% (w/v) trichloracetic acid and then with lipid solvents. In the experiments with ^{14}C-formate and ^{15}N-glycine, RNA was extracted from the tissue residue by "procedure 2" of DAVIDSON AND SMELLIE[10] and precipitated with ethanol. The protein residue was incubated with *N* KOH at 37° for 18 h to remove any residual RNA and was then precipitated by addition of TCA to a concentration of 10% (w/v). The precipitate was finally washed with ethanol and ether and dried. A similar procedure was adopted in experiments with ^{35}S-methionine.

The precipitated RNA was dissolved in water by addition of the minimum amount of alkali and deproteinised by shaking repeatedly with 5:1 chloroform/octanol mixture[25]. It was finally precipitated by the addition of 10 vol. glacial acetic acid, washed with ethanol and ether and dried.

* Beit Memorial Research Fellow.

References p. 565.

In the experiments with ^{14}C-formate, a portion of RNA was hydrolysed with alkali and separated into the component nucleotides by ionophoresis on paper[9].

For the isolation of nuclei the nuclear fraction of the original sucrose dispersion was treated with ice cold 0.05 *M* citric acid and homogenised by rapid stirring for 5 min in a M.S.E. Nelco blendor fitted with a rotating paddle. This process resulted in the disruption of any whole cells which had survived from the original homogenate. From this dispersion nuclei were isolated by the citric acid procedure as previously described[20].

The isolated nuclei were suspended in water and treated with 0.1 *N* NaOH to pH 8.0. A jelly-like mass was obtained and to this was added sufficient *M* NaCl to give a final concentration of 0.14 *M*. Insoluble material was centrifuged down and washed with 0.14 *M* NaCl. The supernatant fluid and washings were acidified with 0.1 *N* HCl and two vol. ethanol added. The precipitate so formed was centrifuged down, washed with ethanol and ether and dried. It contained most of the RNA of the nuclei and will be referred to as *ribonucleoprotein*.

The material insoluble in 0.14 *M* NaCl was suspended in *M* NaCl (pH 8) in which a considerable proportion dissolved. Material insoluble in *M* NaCl was centrifuged down, washed with 65% (v/v) ethanol-water, then with absolute ethanol, ether and dried (*insoluble nucleoprotein*). The solution in *M* NaCl was acidified with 0.1 *N* HCl and treated with 2 vol. ethanol. The precipitate was washed with ethanol and ether and dried (*soluble nucleoprotein*).

The soluble nucleoprotein was extracted with 3 successive portions of 0.2 *N* HCl to remove histone. The solution of histone was made alkaline with ammonia and 2 vol. ethanol added. After standing overnight at 0°, histone was centrifuged down and washed with ethanol and ether and dried. The residue remaining after extraction of histone was suspended in 0.1 *N* NaOH and saturated with NaCl to dissociate DNA from protein. After standing for 48 h at 0° the suspension was treated with chloroform-octanol according to the method of SEVAG *et al.*[25] and the protein separated from the aqueous phase containing the DNA. The protein was removed from the chloroform phase by addition of ethanol and was finally washed with 65% (v/v) ethanol-water, then with ethanol and ether and dried, (*fraction B*). The DNA was precipitated by acidification of the aqueous phase and addition of 2 vol. ethanol. It was centrifuged down, washed free of NaCl with 65% (v/v) ethanol-water, then with ethanol and ether and dried.

The insoluble nucleoprotein was treated with 0.2 *N* HCl to remove histone and was then put through the treatment with NaOH, saturated NaCl and chloroform-octanol as described above to yield a further quantity of DNA and a protein *fraction C*.

The ribonucleoprotein was suspended in saturated NaCl at pH 8 and treated with chloroform-octanol as described above to yield RNA and a protein *fraction A*.

The DNA and RNA were incubated in 0.3 *N* NaOH at 37° for 18 h and the digest acidified, treated with 2 vol. ethanol and centrifuged.

From the RNA fraction this process yielded a solution of ribonucleotides.

From the DNA fraction a precipitate of DNA free from RNA and protein was finally obtained.

^{14}C *assay*

The material to be assayed was dissolved in water with the addition, where necessary, of the minimum amount of 0.1 *N* NaOH. For some of the proteins mild warming of the mixture was necessary. A measured portion of the solution was pipetted on to a nickel-plated flat planchette 15 mm diameter and dried with the aid of an infrared lamp to give a layer of infinite thinness. Other portions of the same solution were used for estimation of nitrogen and phosphorus.

Counting was carried out in a stream of methane gas in a flow counter attached through a linear amplifier Type 1008A to a scaling unit Type 1009A (E.K. Cole). Results are expressed for proteins as counts per minute per 100 μg protein N, for nucleotides as counts per minute per μM, and for whole RNA and DNA as counts per minute per 100 μg P.

^{35}S *assay*

Weighed portions of the proteins after removal of the nucleic acids were ashed with oxygen in a stainless steel bomb and sulphur estimated as benzidine sulphate[14]. Portions of the material were then transferred to a planchette for counting.

The remainder of the protein was dissolved in the minimum of dilute alkali, if necessary with the aid of heat. One portion of the solution was used for nitrogen estimation and a second was evaporated to dryness on a planchette for counting as for ^{14}C. Results are expressed as counts per minute per 0.1 μg S and also as counts per minute per 10 μg protein N.

^{15}N *assay*

Heavy nitrogen was determined in a Metropolitan Vickers Mass Spectrometer after conversion of organic nitrogen to ammonium salt by Kjeldahl digestion and distillation and liberation of nitrogen gas by treatment with hypobromite in SPRINSON AND RITTENBERG[28] tubes.

References p. 565.

RESULTS

The incorporation of ^{14}C-formate and ^{15}N-glycine into the RNA of nuclei and of cytoplasmic fractions and into nuclear DNA is shown in Table I. With both isotopes incorporation into DNA is much less extensive than into RNA, and with ^{15}N-glycine is almost negligible. In all cases incorporation into nuclear RNA is considerably greater than into the RNA of any of the cytoplasmic fractions, and the ratio of incorporation into nuclear RNA and RNA of whole cytoplasm is approximately the same for ^{15}N-glycine and for ^{14}C-formate at 16 h.

TABLE I

Incorporation of ^{14}C-formate and ^{15}N-glycine into RNA and DNA of resting rat liver. ^{14}C-formate was administered by intramuscular injection in a dose of 80–100 μc/100 g body weight 4 or 16 h prior to killing. ^{15}N-glycine was administered by intraperitoneal injection 24, 22 and 20 h before killing, in a dose of 50 mg glycine (20.92 atom % excess ^{15}N) per injection.

	^{14}C-formate		*^{15}N-glycine*
	4 h counts/min 100 μg P	*16 h counts/min 100 μg P*	*Atom % excess ^{15}N*
RNA			
Whole cytoplasm	1976	1110	0.166
Mitochondria	1340	980	0.155
Microsomes	1379	825	0.151
Cell sap	2005	1065	0.233
Nuclei	3140	2770	0.496
DNA	400	504	0.007

Of the cytoplasmic fractions, incorporation is greatest in the cell sap in all cases while the two particulate fractions are very similar. The relative uptakes of ^{14}C-formate into the RNA's from different parts of the cell are again brought out in Table II which shows values for the individual nucleotides. In all cases adenylic acid shows the highest value, guanylic acid gives values considerably lower, while incorporation into uridylic acid is almost negligible. Cytidylic acid on the other hand shows low values for all cytoplasmic RNA's but a value intermediate between that for adenylic and guanylic acids in nuclear RNA. This high value has been confirmed by our colleague Dr. S. C. FRAZER by examination of the cytosine separated by paper chromatography.

TABLE II

Incorporation of ^{14}C-formate into the nucleotides of RNA in nuclei and cytoplasmic fractions of resting rat liver. The formate was administered in a dose of 80 μc/100 g body weight 16 h before killing.

	counts/min μM				
	Whole cytoplasm	*Mitochondria*	*Microsomes*	*Cell Sap*	*Nuclei*
RNA					
Adenylic acid	349	298	328	370	1105
Guanylic acid	136	133	126	145	478
Cytidylic acid	23	37	7	28	598
Uridylic acid	23	27	25	12	97

References p. 565.

The uptake of all three isotopes into nuclear and cytoplasmic proteins is shown in Table III, which shows that incorporation into the proteins of the microsomes is in general higher than into any other cytoplasmic proteins and that incorporation is of the same order for nuclear proteins as for cytoplasmic proteins. With ^{15}N-glycine, however, fraction A, like nuclear RNA, with which it is associated, shows a high uptake whereas the value for histone is low. In general, incorporation of ^{15}N into cytoplasmic proteins is of the same order as that into cytoplasmic RNA's although the value for microsomal protein is considerably higher than that for microsomal RNA.

TABLE III

Incorporation of ^{14}C-formate, ^{15}N-glycine and ^{35}S-methionine into the proteins of resting rat liver nuclei and cytoplasmic fractions. ^{14}C-formate was administered by intramuscular injection 4 h or 16 h before killing, in a dose of 80–100 μc/100 g body weight. ^{15}N-glycine (20.92 atom % excess ^{15}N) was given intraperitoneally in 3 injections of 50 mg each, 24, 22 and 20 h before killing. ^{35}S-methionine was given by intramuscular injection in doses of 62.5 μc/100 g body weight 6 and 4 h before killing.

Type of protein	*^{14}C-formate*		*^{15}N-glycine*	*^{35}S-methionine*	
	counts/min 100 μg protein N 4 h	*counts/min 100 μg protein N 16 h*	*Atom % excess ^{15}N*	*counts/min 0.1 μg S*	*counts/min 10 μg protein N*
Cytoplasm					
Whole cytoplasm	—	509	0.182	120	699
Mitochondria	296	414	0.130	136	778
Microsomes	575	510	0.212	165	898
Cell sap	381	689	0.160	107	615
Nuclei					
Histone	630	318	0.061	*	*
Fraction A	550	256	0.303	132	792
Fraction B	500	377	0.144	120	769
Fraction C	670	492	0.151	144	677

* The amount of sulphur in histone was too low to permit of precise estimation.

DISCUSSION

We have previously confirmed[27] that the incorporation of ^{32}P into the cytoplasmic RNA of resting rat liver is considerably greater than into the DNA. The present experiments show a similar trend with other isotopes. Thus, while appreciable amounts of the carbon of ^{14}C-formate are incorporated into nuclear DNA, incorporation into the RNA of whole cytoplasm is 5 times greater 4 h after administration and twice as great 16 h after administration. A similar 2:1 ratio in the uptake of ^{14}C-formate has been recorded by TOTTER, VOLKIN AND CARTER[30] for the adenylic acid of RNA and DNA 16 h after administration, while a 3:1 ratio was found by PAYNE, KELLY AND JONES[22] for whole RNA and DNA 4 h after administration of ^{14}C-formate.

Isotopic glycine can also be used to label both nucleic acids. ELWYN AND SPRINSON[11], LEPAGE AND HEIDELBERGER[19] and TYNER, HEIDELBERGER AND LEPAGE[31,32] using (2-^{14}C)-glycine found incorporation into DNA almost as great as into RNA. With ^{15}N-glycine FURST AND BROWN[12] found incorporation into RNA purines to be twice as great as into DNA purines while an even greater difference, comparable with that found by ourselves for whole RNA and DNA (Table I), has been recorded by DALY, ALLFREY AND MIRSKY[8].

References p. 565.

It has been shown that the incorporation of ^{32}P into the RNA of resting rat liver nuclei is much greater than into the RNA of any of the cytoplasmic fractions[2,21,16,27]. This is confirmed by the experiments with ^{14}C-formate and ^{15}N-glycine shown in Table I although, in general, the difference is more pronounced with ^{32}P than with ^{14}C-formate or ^{15}N-glycine. This is in agreement with the observations of ANDERSON AND AQUIST[1] who compared ^{32}P with ^{15}N-orotic acid. A higher uptake of ^{15}N-glycine by the purines of nuclear RNA than of cytoplasmic RNA in resting rat liver is recorded by BERGSTRAND, ELIASSON, HAMMARSTEN, NORBERG, REICHARD AND VON UBISCH[3] while PAYNE *et al.*[22] using ^{14}C-formate in mice obtained results similar to our own with rats for the relative incorporations of ^{14}C by nuclear and cytoplasmic RNA.

Of the individual ribonucleotides adenylic acid shows the highest incorporation of ^{14}C-formate in all cytoplasmic RNA's and in nuclear RNA. This high activity of adenylic acid is also shown with ^{32}P[27] and is in agreement with the general pattern of results obtained by TOTTER *et al.*[30] and GOLDTHWAITE[13] with ^{14}C-formate. Guanylic acid gives figures less than half those for adenylic acid while the pyrimidine nucleotides in the cytoplasmic RNA's show very little incorporation at all. While only slight incorporation of ^{14}C-formate takes place in the uridylic acid of RNA from both nucleus and cytoplasm, the uptake of ^{14}C-formate by nuclear, but not by cytoplasmic, cytidylic acid gives an unusually high figure which was confirmed by separation of the cytosine by chromatography. The reason for this is at present obscure.

In our experiments with ^{32}P it was found that incorporation into the RNA of the cytoplasmic fractions was greatest in the cell sap[27]. The particulate fractions showed little difference between microsomes and mitochondria although at short time intervals the latter tended to show slightly higher values than the former. A similar close relationship between mitochondria and microsomes has been recorded by BARNUM AND HUSEBY[2] using mouse liver, whereas JEENER AND SZAFARZ[16] using conditions for the separation of cytoplasmic fractions considerably different from ours, obtained greater incorporation into the microsomes than into the mitochondria. Our results with ^{14}C-formate and ^{15}N-glycine confirm the higher activity of the RNA of the cell sap than of the particles and also the similarity between the RNA's of microsomes and mitochondria. On the other hand, the pattern obtained for incorporation into the proteins of the cytoplasmic fractions clearly shows a higher activity for the microsomes with ^{14}C-formate, ^{15}N-glycine and ^{35}S-methionine. A similar high incorporation into the proteins of microsomes as compared with other cytoplasmic fractions has been found for liver tissue by TYNER *et al.*[31] using ^{14}C-glycine in the rat, by HULTIN[15] using ^{15}N-glycine in the chick, by KELLER[17] using (1-^{14}C)-leucine in the rat, by LEE, ANDERSON, MILLER AND WILLIAMS[18] using ^{35}S-cystine in the rat, by BORSOOK, DEASY, HAAGEN-SMIT, KEIGHLEY AND LOWY[4,5] using (1-^{14}C)-glycine, lysine and leucine in guinea pig liver homogenates, and by SIEKEVITZ[26] using (1-^{14}C)-alanine in rat liver homogenates. The importance of this high incorporation into microsomes in relation to protein synthesis has been discussed by BRACHET[6] and by CHANTRENNE[7].

Incorporation into nuclear proteins is of the same magnitude as into cytoplasmic proteins, and with ^{14}C-formate or ^{35}S-methionine the figures for all nuclear proteins are of the same order. With ^{15}N-glycine, on the other hand, a high value is found for fraction A and a low value for histone. This is in confirmation of the results obtained in mice by DALY *et al.*[8], who found a much lower figure for the incorporation of ^{15}N-glycine into histone than into "residual protein". Their "residual protein" gave values of the

References p. 565.

same order as total cytoplasmic protein and presumably corresponded in composition with the chromosomin of STEDMAN AND STEDMAN[29] in being essentially the non-histone protein of the nucleus. In our experiments this material is represented by fractions A, B and C, which together show an incorporation similar to that of whole cytoplasmic protein.

The general pattern which emerges from these results is that the high metabolic activity of nuclear RNA demonstrated with ^{32}P has been confirmed with the aid of ^{14}C and ^{15}N, as also has the low activity of DNA. The uptake of ^{32}P and ^{14}C by RNA is appreciably greater in the cell sap than in either microsomes or mitochondria which closely resemble each other, but incorporation of ^{14}C, ^{15}N and ^{35}S into the cytoplasmic proteins is most pronounced in the microsomes. The metabolic activities of the RNA's and proteins in the cytoplasmic fractions do not appear, therefore, to be identical. The nuclear proteins have an activity comparable with that of the cytoplasmic proteins and must therefore be presumed to take an active part in metabolic processes even in non-dividing cells.

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SUMMARY

1. The incorporation of isotopes into the nucleic acids and proteins of rat liver cell nuclei and cytoplasmic fractions has been studied with the aid of ^{15}N-glycine, ^{14}C-formate and ^{35}S-methionine.

2. In confirmation of earlier results with ^{32}P it was found with ^{15}N-glycine and ^{14}C-formate that incorporation into nuclear DNA was slow but that nuclear RNA showed a high rate of incorporation which exceeded that of the RNA of any of the cytoplasmic fractions. Of these fractions, the RNA of the cell sap showed a higher incorporation than did the RNA of the cytoplasmic granules. No great differences were found between mitochondria and microsomes.

3. No great differences in incorporation into protein were found between nuclei and cytoplasm. In the cytoplasm the microsomal protein showed a higher incorporation than did the protein of mitochondria or cell sap. When ^{15}N-glycine was used, incorporation into the nuclear proteins was found to be low in histone but high into the nuclear protein which accompanied the RNA.

RÉSUMÉ

1. On a étudié l'incorporation d'isotopes dans les acides nucléiques et dans les protéines des noyaux des cellules du foie de rat, ainsi que dans celles des fractions cytoplasmiques, à l'aide de ^{15}N-glycine, de ^{14}C-formate et de ^{35}S-méthionine.

2. En confirmation de résultats antérieurs obtenus avec le ^{32}P, on a trouvé, au moyen de ^{15}N-glycine et de ^{14}C-formate, que l'incorporation dans le DNA nucléaire est basse, mais que le RNA du noyau présente un taux élevé d'incorporation, qui dépasse celui du RNA de toutes les fractions cytoplasmiques. Parmi ces fractions, le RNA du suc cellulaire manifeste une incorporation plus haute que le RNA des granules cytoplasmiques. On n'a pas trouvé de différences importantes entre le RNA des mitochondries et celui des microsomes.

3. On n'a pas trouvé non plus de différences importantes entre les noyaux et le cytoplasme en ce qui concerne l'incorporation dans les protéines. Dans le cytoplasme, les protéines des microsomes

References p. 565.

montrent une incorporation plus forte que celles des mitochondries et celles du suc cellulaire. En employant la ^{15}N-glycine, l'incorporation dans les protéines nucléaires s'est révélée basse dans le cas de l'histone, mais haute dans celui des protéines nucléaires extraites avec le RNA.

ZUSAMMENFASSUNG

1. Es wurde der Einbau von Isotopen in die Nucleinsäuren und in die Proteine der Zellkerne und in die Bruchstücke des Zellplasmas von Rattenleber mit Hilfe von ^{15}N-Glycin, ^{14}C-Formiat und ^{35}S-Methionin studiert.

2. Übereinstimmend mit früheren Ergebnissen mit ^{32}P fand man, dass der Einbau bei ^{15}N-Glycin und ^{14}C-Formiat in die Kern-DNS langsam stattfand, aber dass der Einbau in die Kern-RNS mit grosser Geschwindigkeit vor sich ging, der diejenige der RNS in allen Bruchstücken des Zellplasmas übertraf. Von diesen Bruchstücken zeigte die RNS des Zellsaftes eine grössere Einbaugeschwindigkeit als die RNS der Körner des Zellplasmas. Keine grossen Unterschiede wurden zwischen den Mitochondrien und den Mikrosomen gefunden.

3. Es wurden keine grossen Unterschiede zwischen den Zellkernen und dem Cytoplasma beim Einbau in die Proteine gefunden. Im Cytoplasma zeigte das Protein der Mikrosomen einen höheren Einbau als das Protein der Mitochondrien oder des Zellsaftes. Bei der Verwendung von ^{15}N-Glycin wurde gefunden, dass der Einbau in die Kern-proteine bei Histon langsam, aber schnell bei den die RNS begleitenden Kernproteinen stattfand.

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