The Plasma Transport and Metabolism of Retinoic Acid in the Rat

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(Received 11 December 1972)

The transport of retinoic acid in plasma was examined in vitamin A-deficient rats maintained on small doses of radioactively labelled retinoic acid. After ultracentrifugation of serum adjusted to density 1.21, most of the radioactivity (83%) was associated with the proteins of density greater than 1.21, and not with the serum lipoproteins. Gel filtration of the labelled serum on Sephadex G-200 showed that the radioactive label was associated with protein in the molecular-weight range of serum albumin. On polyacrylamide-gel electrophoresis almost all of the recovered radioactivity migrated with serum albumin. Similar results were obtained with serum from a normal control rat given a single oral dose of [14C]retinoic acid. These findings indicate that retinoic acid is transported in rat serum bound to serum albumin, and not by retinol-binding protein (the specific transport protein for plasma retinol). Several tissues and the entire remaining carcase of each rat were extracted with ethanol–acetone to determine the tissue distribution of retinoic acid and some of its metabolites. The total recovery of radioactive compounds in the entire body of the rat was about 7–9 μg, representing less than 5% or 10% respectively of the total administered label in the two dosage groups studied. The results confirm that retinoic acid is not stored in any tissue. Most of the radioactive material was found in the carcase, rather than in the specific tissues analysed. Two-thirds of the radioactivity in the carcase appeared to represent unchanged retinoic acid. Of the tissues examined, the liver, kidneys and intestine had relatively high concentrations of radioactive compounds, whereas the testes and fat-pads had the lowest concentrations.

It is well established that retinoic acid demonstrates selective vitamin A biological activity. Thus, rats which are maintained on a vitamin A-deficient diet supplemented with retinoic acid become blind (Dowlow & Wald, 1960) and sterile (Thompson et al., 1964), but are otherwise in a good general state of health and grow at a normal rate. Retinoic acid differs from other compounds with vitamin A biological activity in that it cannot be converted into retinol (Dowlow & Wald, 1960; Krishnamurthy et al., 1963). Very little information is available on the metabolic history of retinoic acid. Orally administered retinoic acid is mainly adsorbed by the portal route (Fidge et al., 1968) and then rapidly metabolized, largely to more-polar compounds excreted in the urine (Geison & Johnson, 1970) and the bile (Zachman et al., 1966; Fidge et al., 1968). The major metabolite in rat bile has been identified as retinoyl β-glucuronide (Dunagin et al., 1965). Animals do not store retinoic acid in the liver or in other tissues, even after large doses of the compound, although very small amounts of retinoic acid have been detected transientsly in plasma and in other tissues (liver, kidney, intestine) after oral administration (Dowlow & Wald, 1960; Krishnamurthy et al., 1963; Geison & Johnson, 1970; Jurkowitz, 1962; Nelson et al., 1965; Morgan & Thompson, 1967; Zile & DeLuca, 1968; Redfearn, 1960).

Vitamin A normally circulates in plasma as retinol bound to a specific protein, retinol-binding protein. Human (Kanai et al., 1968) and rat retinol-binding protein (Muto & Goodman, 1972) have been isolated and partially characterized. In both species retinol-binding protein has a molecular weight close to 20000, and circulates in the form of a protein–protein complex, together with a larger protein with pre-albumin mobility on electrophoresis. Human plasma retinol-binding protein can bind retinoid acid almost as effectively as it can bind retinol (Goodman & Raz, 1972). The manner in which retinoic acid is transported in plasma in vivo is, however, not known.

We now report studies designed to characterize the transport of retinoic acid in plasma in vitamin A-deficient rats maintained on small doses of retinoic acid. Information is also presented about the tissue concentrations and distribution of retinoic acid and some of its metabolites in these rats.

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Methods

This study was carried out concurrently with a study, previously reported (Muto et al., 1972), of the regulation of retinol-binding-protein metabolism by nutritional vitamin A status in the rat. The rats employed in the present study comprised some of the animals of the retinoic acid-treated groups of rats in the previously reported study.

Six male weanling rats of the Holtzman strain were depleted of their vitamin A stores by feeding a diet free of vitamin A (Muto et al., 1972). The depletion of the vitamin A stores was considered nearly complete by the 25th day in view of the very low serum vitamin A concentrations (about 6 µg/100 ml). The rats were then divided into two groups of three each, designated as group A and group B (and included in groups 4 and 5, respectively, in the study reported by Muto et al. (1972)). Each rat in group A was given 14 µg of retinoic acid/day, a dose that sustained a lesser than normal rate of growth. Each rat in group B was given 28 µg/day, a dose that supported growth comparable with that of normal control rats (Muto et al., 1972). The retinoic acid, dissolved in peanut oil which contained 0.1% hydroquinone as an antioxidant, was given orally once each day with a calibrated dropping pipette.

On the 53rd day of the study 14C-labelled retinoic acid was substituted for the previously administered unlabelled retinoic acid. Each rat in both groups received 14 µg of [6,7-14C]retinoic acid (0.33 µCi)/day. Each rat of group B was given an additional 14 µg of unlabelled retinoic acid/day to maintain the total daily intake at 28 µg. The labelled compound was administered for 8 days to try to approach a steady state.

Approximately 24 h after the last dose of [14C]-retinoic acid had been given the rats were anaesthetized with ether and blood was withdrawn from the abdominal aortae into syringes. From each rat the liver, kidneys, epididymal fat-pads, small intestine, testes, and adrenals were removed. The tissues were rinsed, blotted dry, weighed, sliced, and extracted in ethanol–acetone (1:1, v/v). Extraction was carried out by homogenizing the tissue with ethanol–acetone (1:1, v/v) in a Waring Blender, and then adjusting the homogenate volume to 50 ml. The carcase and remaining viscera were ground and similarly extracted with 3.5 litres of the ethanol–acetone. Blood samples were centrifuged at 700 g (r, 15 cm) for 30 min at 4°C and the sera collected. A portion (4 ml) of each serum sample was extracted with 4.5 ml of the ethanol–acetone. Measured portions of each extract were assayed for radioactivity.

 Portions of the tissue extracts from one of the rats in group A were processed to separate acidic and non-acidic lipids by the method of Borgström (1952). For each sample, the ethanol–acetone extract was evaporated to dryness under N2 and the residue was suspended in 10 ml of hexane and extracted with an equal volume of 0.1 M NaOH inaq. 50% (v/v) ethanol. After the removal of the hexane phase, which contained the non-acidic lipids, the acidic lipids were recovered by acidification of the ethanolic NaOH with 1 M HCl and extraction with hexane. The hexane solutions of non-acidic and of acidic lipids and a portion of the ethanol–water were assayed for radioactivity.

A composite sample of 6 ml of fresh serum (1 ml from each rat) was adjusted to density 1.21 with a solution of KBr, and lipoproteins were separated as a single fraction by centrifugation for 47 h at 105000 g (r, 5.9 cm) in the 40 rotor of a Spinco model L ultracentrifuge. The centrifuge tube was sliced in the clear zone below the floating lipoprotein layer, to obtain two fractions comprising the lipoproteins (density less than 1.21) and the other serum proteins with density greater than 1.21. These fractions were extracted with 10 vol. of the ethanol–acetone and appropriate portions of the extracts were assayed for radioactivity.

Samples of the 14C-containing sera were fractionated by gel filtration on columns of Sephadex G-200 and by preparative polyacrylamide-gel electrophoresis, using techniques described in previous publications from this laboratory (Kanai et al., 1968; Muto & Goodman, 1972; Raz & Goodman, 1969).

The eluted fractions were combined into pools (see the Results section) and portions of the pools were extracted with 20 vol. of chloroform–methanol (2:1, v/v). The extracts were split into two phases by addition of 5 mM H2SO4, and measured portions of each chloroform phase (comprising the total lipid extract of the sample) were assayed for radioactivity.

In a separate experiment, serum was obtained from a normal adult rat given a single oral dose of 0.66 µCi (28 µg) of [14C]retinoic acid. This rat had been receiving 120 µg of vitamin A (as retinyl esters)/day. Blood was drawn 6 h after the administration of the [14C]retinoic acid, and the serum was collected and subjected to preparative polyacrylamide-gel electrophoresis.

For assay of radioactivity, samples were evaporated to dryness under N2 and the residues each dissolved in 15 ml of 0.5% diphenyloxazole in toluene. The samples were assayed in a Packard liquid-scintillation counter, model 3155. When appropriate, corrections for quenching were made by use of an automatic external standard of 32P-Ra. The counting efficiency of this system was about 82% for 14C.

The retinol-binding-protein content of the various pooled samples of the preparative polyacrylamide-gel electrophoresis experiments was determined by radioimmunoassay (Muto et al., 1972).

The [6,7-14C]retinoic acid (sp. radioactivity 23.5 µCi/mg) was a generous gift of Hoffmann–La Roche, Basle, Switzerland. The radiochemical purity of the
labelled retinoic acid was determined in our laboratory at the time of this study to be greater than 90%, both by differential solvent extraction (Borgström, 1952) and by thin-layer chromatography on silica gel with methanol–benzene (3:17, v/v) as solvent.

Results

Transport of retinoic acid in serum

After ultracentrifugation of a composite sample of serum at density 1.21, 83% of the serum radioactivity was recovered in the 'bottom' fraction containing the proteins of hydrated density greater than 1.21. The floating lipoprotein fraction contained only 17% of the total radioactivity.

The 14C-containing rat serum was fractionated by gel filtration; the results are illustrated in Fig. 1. Most (approx. 87%) of the radioactivity in the eluate from the column was found in association with the peak of protein that represented serum albumin and proteins of similar molecular size (approx. 50000–80000 molecular-weight range). The recovery of radioactivity applied to the Sephadex column was about 95%.

Labelled rat serum was fractionated by preparative polyacrylamide-gel electrophoresis, particularly to separate serum albumin from the α-migrating retinol-binding protein. Fig. 2 presents results obtained with a normal control rat. The results clearly show that the radioactivity migrated in association with serum albumin. Virtually no radioactivity migrated in the region associated with retinol-binding protein. In this rat whose serum contained vitamin A the retinol-binding-protein region was easily identified by the specific fluorescence of holo-(retinol-binding protein) (Muto & Goodman, 1972; Goodman & Leslie, 1972). The recovery of label from this gel was about 70%. A very similar migration pattern was obtained with serum from a rat in group A.

Tissue distribution of retinoic acid and its metabolites

The observed content of radioactivity in the various tissues of the rats in groups A and B are shown in the first two columns of Table 1. The liver, small intestine, and kidneys showed the highest contents of the radioactive label. Serum and adrenal glands had intermediate values, whereas adipose tissue and testes had relatively low amounts of the label. Except for small intestine, the tissues of the rats in group A (14 µg of retinoic acid/day) contained higher amounts of radioactivity than did those of the rats in group B (28 µg/day).

The total amounts of labelled retinoic acid and its metabolites found in the tissues of the two groups of rats are shown in the last two columns of Table 1. The total recovery of radioactive compounds in the entire body of the rat was about 7–9 µg in both groups of animals. Most (70–80%) of the radioactive material was found in the 'carcass', which mainly consisted of skeletal muscle (45.5% of body weight (Castor et al., 1956)) and of other skeletal and connective tissue. The livers contained about 10% of the radioactive material found, and lesser amounts were found in the other tissues examined.

Information about the nature of the radioactive compounds found in the tissues of one of the rats of group A is presented in Table 2. Unchanged retinoic acid would be expected to be recovered in the hexane solution of acidic lipids after solvent partition. Thus, 92.5% of the radioactivity of an extract of the [14C]retinoic acid solution given to the rats was recovered in the acidic-lipid fraction. Approximately two-thirds of the total radioactivity found in the rat's body partitioned as an acidic lipid, and presumably was present as unchanged retinoic acid. In particular, 68% of the 14C present in the carcass extract and 58% of that in the liver extract appeared to be present as retinoic acid. In the serum and kidney extracts almost one-half of the radioactivity partitioned as an acidic lipid (unchanged retinoic acid), whereas in the other tissues examined retinoic acid appeared to represent only 25–36% of the 14C-labelled materials present. Significant amounts of labelled compounds

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weight of the in each details For 1.

Table which fat, collected at a of the other tissues removal The sample indicated as (Castor et al., 1965). The Liver was 2.5ml sample, was 2.5ml protein/ml of peak detected. pH 8.1, buffer, was 2.5ml protein column 10cm was 2.5ml of 7% (w/v) polyacrylamide gel at a constant 400V and at approx. 21mA. Fractions of 5.6ml were collected at a flow rate of 34ml/h. Fractions were combined into pools for assay of 14C radioactivity (d.p.m./ml), as indicated by the bars in the Figure. —— represents E280, and ■ represents the radioactivity. The main peak of protein eluted (fractions 41–46) represents serum albumin. The fluorescence of protein-bound retinol [holo-(retinol-binding protein)], with an excitation maximum of 334nm and emission maximum of 460nm is also shown (—). The pool from the fluorescent fractions (71–88) was found to contain 1.44μg of retinol-binding protein/ml by radioimmunoassay (Muto et al., 1972). This was the only pool in which retinol-binding protein was detected.

Fig. 2. Preparative polyacrylamide-gel electrophoresis of rat serum containing [14C]retinoic acid

The sample was 2.5ml of serum obtained from a normal adult rat 6h after a single dose of [14C]retinoic acid. The sample, which had been previously dialysed against 2 litres of a 49.5mm-Tris–384mm-glycine–0.5mm-HCl buffer, pH 8.1, was applied to the gel as a 5% (w/v) sucrose solution. Electrophoresis was conducted on the 10cm column of 7% (w/v) polyacrylamide gel at a constant 400V and at approx. 21mA. Fractions of 5.6ml were collected at a flow rate of 34ml/h. Fractions were combined into pools for assay of 14C radioactivity (d.p.m./ml), as indicated by the bars in the Figure. —— represents E280, and ■ represents the radioactivity. The main peak of protein eluted (fractions 41–46) represents serum albumin. The fluorescence of protein-bound retinol [holo-(retinol-binding protein)], with an excitation maximum of 334nm and emission maximum of 460nm is also shown (—). The pool from the fluorescent fractions (71–88) was found to contain 1.44μg of retinol-binding protein/ml by radioimmunoassay (Muto et al., 1972). This was the only pool in which retinol-binding protein was detected.

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Table 1. Tissue distribution of radioactivity and labelled retinoic acid and its metabolites in rats given [14C]retinoic acid

For details see the text. Values are given as means ± S.E.M. The total ng of retinoic acid and its metabolites found in each tissue was calculated from the observed content of 14C (d.p.m./g of tissue) together with the measured weight of the tissue and the known specific radioactivity of the original [14C]retinoic acid fed to the rat. Epididymal fat, which was analysed, was assumed to be representative of body depot fat. Depot fat was taken as 7.08% (Castor et al., 1956), and plasma volume as 3.5% of the body weight, as in a previous study (Goodman et al., 1965). The values for serum are given as d.p.m./ml and the values for the carcase represent all tissues left after removal of the other tissues listed.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Radioactivity (d.p.m./g of tissue)</th>
<th>Amount of retinoic acid and its metabolites (total ng/tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
</tr>
<tr>
<td>Liver</td>
<td>3652 ± 323</td>
<td>2543 ± 338</td>
</tr>
<tr>
<td>Kidneys</td>
<td>2117 ± 307</td>
<td>1427 ± 83</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2640 ± 229</td>
<td>2759 ± 708</td>
</tr>
<tr>
<td>Fat</td>
<td>431 ± 19</td>
<td>216 ± 16</td>
</tr>
<tr>
<td>Testes</td>
<td>257 ± 38</td>
<td>164 ± 12</td>
</tr>
<tr>
<td>Adrenals</td>
<td>1518 ± 196</td>
<td>919 ± 144</td>
</tr>
<tr>
<td>Serum</td>
<td>1611 ± 190</td>
<td>841 ± 71</td>
</tr>
<tr>
<td>Carcase</td>
<td>1068 ± 197</td>
<td>475 ± 102</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Table 2. Characterization of the radioactive compounds in tissues from rat given [14C]retinoic acid

For details see the text.

% Distribution of Compounds more polar than retinoic acid

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Non-acidic lipids</th>
<th>Acidic lipids</th>
<th>(ethanol–water phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>18.6</td>
<td>58.3</td>
<td>23.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>24.0</td>
<td>45.5</td>
<td>30.5</td>
</tr>
<tr>
<td>Small intestine</td>
<td>24.8</td>
<td>33.1</td>
<td>42.1</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>61.5</td>
<td>25.2</td>
<td>13.3</td>
</tr>
<tr>
<td>Testes</td>
<td>40.4</td>
<td>36.5</td>
<td>23.1</td>
</tr>
<tr>
<td>Carcase</td>
<td>16.9</td>
<td>68.1</td>
<td>15.0</td>
</tr>
<tr>
<td>Serum</td>
<td>5.0</td>
<td>46.4</td>
<td>48.6</td>
</tr>
<tr>
<td>Retinoic acid solution</td>
<td>1.5</td>
<td>92.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Serum collected at 6h</td>
<td>5.2</td>
<td>69.7</td>
<td>25.1</td>
</tr>
</tbody>
</table>

more polar than retinoic acid (recovered in the ethanol–water phase) were found in the extracts of all of the tissues. These unidentified compounds presumably represented labelled metabolites of retinoic acid, such as (and including) retinoyl β-glucuronide. The largest proportions of labelled metabolites more polar than retinoic acid (40–50% of the total radioactivity) were found in the extracts of serum and small intestine. The smallest proportions of labelled metabolites more polar than retinoic acid (13–15% of the total 14C) were found in the extracts of the carcase and the epididymal fat. Relatively large proportions of the radioactivity present in the testes and the epididymal fat-bodies was present as non-acidic neutral lipids. This material was not characterized further, but might have represented retinoic acid present in ester linkage, e.g. as a glyceryl or cholesteryl ester. Alternatively, it might have reflected some reutilization of label after metabolism of retinoic acid.

Most (70%) of the radioactivity present in the serum obtained from the normal control rat given a single dose of [14C]retinoic acid 6h previously was present as unchanged retinoic acid.

Discussion

Retinoic acid is unique in the family of compounds known to have vitamin A activity in that it cannot be converted into retinol (Dowling & Wald, 1960; Krishnamurthy et al., 1963). We recently reported a study of the effects of vitamin A depletion and deficiency, and of repletion, on the amount of serum retinol-binding protein in rats (Muto et al., 1972). Vitamin A-deficient rats had amounts of serum retinol-binding protein approximately one-quarter those of normal control animals. Administration of retinoic acid to vitamin A-deficient rats did not affect amounts of serum retinol-binding protein, whereas the administration of retinyl acetate resulted in a rapid increase in the amount of serum retinol-binding protein. These observations raised the question of the manner in which retinoic acid is transported in plasma.

In the present study, vitamin A-deficient rats were given small oral doses of [14C]retinoic acid, in amount sufficient to maintain good health and normal growth (group B, 28µg/day), or in amount able to sustain a lesser, marginal rate of growth (group A, 14µg/day). Serum samples were collected to characterize the transport vehicle, and tissues were analysed to examine the overall metabolism of retinoic acid.

After ultracentrifugation of a sample of serum adjusted to density 1.21, most of the radioactivity (83%) was associated with the proteins of density greater than 1.21. The lipoproteins, with densities less than 1.21, therefore did not play an important role in the plasma transport of retinoic acid.

Gel filtration of the labelled serum on Sephadex G-200 showed that the radioactive label was associated with protein in the molecular-weight range of serum albumin. Retinol-binding protein in rat whole serum circulates as a protein–protein complex, together with prealbumin-2, with an apparent molecular weight similar to that of serum albumin (Muto & Goodman, 1972). It was, therefore, not possible from the gel-filtration experiment alone to determine whether the labelled retinoic acid was associated with serum albumin or with retinol-binding protein. Rat serum albumin and retinol-binding protein are, however, effectively separated by polyacrylamide-gel electrophoresis. On electrophoresis almost all of the recovered radioactivity migrated with serum albumin, with only traces of radioactivity...
in the region in which retinol-binding protein migrated. These results indicate that the retinoid acid was transported in serum bound to serum albumin, and not by retinol-binding protein. Similar results were also obtained with serum from a normal control rat given a single dose of \(^{14}\)C-retinoic acid.

By using a radioimmunoassay technique, vitamin A-deficient rats given retinoic acid were found to have a mean amount of serum retinol-binding-protein of about 0.6 nmol/ml (Muto et al., 1972); virtually all of this was present as apo-(retinol-binding protein), not containing bound retinol. The maximum concentration of retinoic acid found in serum in this study was 0.124 nmol/ml. The serum, therefore, contained a molar excess of retinol-binding protein relative to retinoic acid, with sufficient binding sites for retinol-binding protein available to accommodate all of the retinoic acid present. From these results it is clear that retinol-binding protein is not the transport vehicle for retinoic acid. Unless a species difference exists, the observation that human retinol-binding protein can effectively bind retinoic acid (Goodman & Raz, 1972) appears to have no physiological significance.

Retinoic acid thus appears to be transported as are other free fatty acids (Goodman, 1958; Fredrickson & Gordon, 1958), bound to serum albumin and presumably in the form of its carboxylate anion. Fidge et al. (1968) previously suggested that retinoic acid was probably absorbed from the intestine into portal blood in the form of the free acid bound to serum albumin. Although the serum samples contained radioactive metabolites other than retinoic acid, the high recovery of label from the columns in association with serum albumin confirms that albumin is the major transport protein for retinoic acid. A similar conclusion, that retinoic acid binds to and is transported by serum albumin, was reported in abstract form by Lehman et al. (1972). From the recoveries of label it would appear, moreover, that albumin may also be the major transport protein for the more polar metabolites of retinoic acid found in serum.

All of the tissues examined contained small but significant amounts of radioactivity. The total recovery of radioactive compounds in the entire body of the animal was, however, very small representing less than 5% of the total \(^{14}\)C administered during the preceding 8 days in the group B rats, and less than 10% in the group A rats. It is, of course, also possible that additional labelled metabolites were present in the animals' tissues that were not soluble in the ethanol–acetone extract, and hence were not detected. These results confirm the previous reports of others that retinoic acid is not stored in any of the animal's tissues. Since the rats in both groups had similar total amounts of retinoic acid in their bodies, the results suggest that near the steady state of retinoic acid metabolism the rat may maintain a small but constant amount of retinoic acid and its metabolites, regardless of the dose given. Most (70–80%) of the radioactive material was found in the carcase, rather than in the specific tissues and organs analysed. Two-thirds of the radioactivity in the carcase appeared to be an acidic lipid, presumably unchanged retinoic acid.

Of the tissues examined, the liver, kidneys and intestine had relatively high concentrations of radioactive compounds, whereas the testes and epididymal fat-pads had much lower concentrations. All of the tissues had significant amounts of labelled compounds more polar than retinoic acid. This was particularly true in the intestine, where more-polar compounds appeared to be present at a higher concentration than retinoic acid itself. Retinoic acid metabolites are known to be excreted into the intestine through the bile duct and to undergo some enterohepatic circulation (Zachman & Olson, 1965; Zachman et al., 1966; Fidge et al., 1968). The fact that group B rats had about twice as much labelled material in their intestines as did group A rats probably reflects the excretion of larger amounts of retinoic acid metabolites via the bile in the rats receiving 28 \(\mu\)g of retinoic acid/day.

We thank Miss B. Adams for expert assistance, and Hoffmann-La Roche, Basle, Switzerland, for the gift of \(^{14}\)C-labelled retinoic acid. This work was supported by grants AM-05968 and HL-14236 (SCR) from the National Institutes of Health, U.S. Public Health Service. J. E. S. was a trainee under grant T1-AM-05397 at the time of this study, and DeW. S. G. is a Career Scientist of the Health Research Council of the City of New York.

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