Effects of Carcinogenic Amines on Amino Acid Incorporation by Liver Systems

I. Secondary Increase in Microsomal Activity after Aminofluorene Treatment

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SUMMARY

Isolated liver microsomes from rats treated in vivo with 2-aminofluorene showed an increased capacity to incorporate labeled amino acids into protein 8–48 hours after the administration of the carcinogen. This effect masked an earlier inhibitory influence of the amine. The stimulation did not appear in adrenalectomized animals, indicating that adrenal hormones might be involved.

The increase in incorporation activity of the microsomes was of the same order of magnitude whether calculated on the basis of microsomal protein or on the basis of microsomal RNA. The difference between preparations from normal and treated animals was diminished but still present when free RNP particles were substituted for microsomes in the incorporation system. The effect was not due to an increased active life span of the microsomes in the incubation system. Concomitantly the proportion of protein-bound isotope increased in the soluble fractions.

The incorporation activity of normal microsomes or RNP particles was increased when cell sap from treated animals was substituted for normal cell sap in the incubation system. Although both cell sap and microsomes from treated animals contributed to the increased incorporation activity, the effect of the microsomes was quantitatively more important.

The endoplasmic membranes of liver cells are the site of enzymatic reactions of great importance in metabolic detoxication. The mechanism of these reactions has been studied in considerable detail in vitro by use of isolated liver microsomes (7, 8, 12, 16, 40, 49). It has been observed that a number of well known liver carcinogens are included in the large group of compounds that are metabolized by the detoxicating microsomal enzymes (7, 20, 35, 40, 49). The primary enzymatic attack is usually oxidative, and, in connection with this step, reactive intermediates or side products may be formed (10, 37, 41, 42, 50). These metabolites manifest themselves by a more or less pronounced tendency to react with proteins and other cell components, primarily in the microsomal membranes but gradually also in other cell fractions (18–21, 34, 36, 40, 50). The level of reactive molecules that is reached under different in vivo or in vitro conditions is determined by a combination of inherent and environmental factors which are still poorly defined (15, 21).

In the last few years the fundamental importance of ribonucleoprotein (RNP) particles in pro-
tein metabolism has been increasingly emphasized. In liver the majority of these particles is intimately associated with the endoplasmic membranes (45). This suggests that an essential part of the cytoplasmic protein formation is performed in close spatial proximity to the detoxication system. It seems reasonable to assume that the anabolic functions of the particles are liable to disturbances whenever the concentration of reactive molecules in and around the membranes surpasses a critical threshold level. It has, in fact, been shown (4, 24) that rat liver slices incubated with the carcinogenic amines 2-aminofluorene (AF), 2-aminonaphthalene (AN), or dimethylnitrosamine have a significantly decreased capacity to incorporate labeled amino acids into protein. A decrease in incorporation activity may also be observed in cell-free rat liver systems prepared a few hours after in vivo treatment with the same carcinogens (8, 24). This effect, which is essentially due to a decreased activity of the microsomes, will be discussed in more detail in a forthcoming paper.2

In experiments with AF and AN, the initial inhibition is soon masked by secondary reactions of an entirely different kind. The incorporation activity of the microsomes rapidly rises, from being abnormally low, far beyond the normal level (25). This enhancement is not correlated with a proportional increase in the RNA content of the microsomes (cf. 14). In the present paper some experiments are described by which this secondary increase in microsomal activity is further illustrated.

MATERIALS AND METHODS

Animals.—Male Wistar rats (150–250 gm.) and male guinea pigs (250–350 gm.) of a local breed were used in the experiments. The animals were maintained on a mixed diet and were starved 16–20 hours before being sacrificed by decapitation.

Chemicals.—AF was synthesized according to Kuhn (31); AAF was prepared by acetylation of AF with acetic anhydride in benzene solution; AN, a preparation from Fluka AG, Buchs, Switzerland, was recrystallized several times from dilute ethanol; Celite (diatomaceous earth) was obtained from Johns Manville Intern. Corp., New York, N.Y.; Δ-hydrocortisone (prednisolone) was purchased from Merck, Sharp & Dohme, Haarlem, Netherlands; and PEP (silver barium salt) from Boehringer GmbH, Mannheim, Germany. Before use PEP was recrystallized (6) and converted to potassium salt by treatment with HCl and K2SO4 in slight excess. Carbamyl phosphate (dilithium salt) and ATP were obtained from Sigma Chemical Co., St. Louis, Mo.; Lubrol W (cetyl alcohol polyoxyethylene condensate) from Imperial Chemical Industries, Manchester, England; pyruvate kinase from Boehringer GmbH; and crystalline bovine plasma albumin from Armour Laboratories, Chicago, Ill. Carbamate kinase was prepared from Streptococcus faecalis and purified according to Mokrasch et al. (38).

The labeled amino acids L-valine-C14 (6.5 mc/mmmole) and L-leucine-C14 (1.5 or 6.5 mc/mmmole) were supplied by the Radiochemical Centre, Amersham, England.

Pretreatment of animals.—AF or AN was finely ground in an agate mortar and suspended in horse serum. In most experiments, half the dose was administered by stomach tube and the other half intraperitoneally. Control animals received corresponding amounts of serum. In some cases AF or AAF was administered by stomach tube only. In these experiments AF was given either as a serum suspension or as a solution in propylene glycol. Δ-hydrocortisone was obtained as a suspension for injection and was given intraperitoneally. Celite was administered intraperitoneally suspended in serum.

For x-radiation a Scandia Intensive apparatus (170 kv., 15 ma., internal filter effect corresponding to 2 mm. of aluminum) was used. The dose was 2,500 r given over a period of 45 minutes.

Incorporation experiments.—The animals were killed by decapitation, and the livers were rapidly excised and minced in an ice-cold medium usually containing 0.25 m sucrose, 0.075 M KCl, 0.01 M MgCl2 and 0.035 M Tris buffer pH 7.8. After repeated washing the mince was homogenized in a loose-fitting all-glass homogenizer in 2.5 volumes of the same medium. To obtain mitochondrial supernatants, nuclei, cell debris, and large cytoplasmic particles were removed by centrifugation at 14,000 × g for 6 min. in a refrigerated International model PR-1 centrifuge.

When mitochondrial fractions were used, the mince was homogenized in 4.0 volumes of medium, and nuclei and cell debris were removed by an initial centrifugation at 600 × g for 5 min. The mitochondrial fractions were then sedimented by centrifugation at 14,000 × g for 6 min. and suspended in medium to a concentration of 4–4.5 mg protein/ml.

For preparation of microsomes and cell sap the mitochondrial supernatants were centrifuged at 105,000 × g for 55 min. RNP particles were prepared from mitochondrial supernatants by treatment with Lubrol W and DOC at an increased potassium concentration, followed by centrifugation through a density gradient, as described by Rendi and Hultin (48). Microsomes and RNP particles

1 T. Hultin and E. Arrhenius, to be published
were suspended in homogenization medium by means of a small, manual homogenizer. The microsome suspensions usually contained 15–20 mg protein and 3–4 mg RNA/ml; the suspensions of RNP particles, 2–3 mg/ml of both protein and RNA; and the cell-sap preparations, 17–23 mg protein/ml.

In the experiments where the relative activity of cell sap preparations was measured in the presence of an excess of microsomes or RNP particles, the microsomes were largely freed from contaminating cell sap by centrifugation through a layer of medium with higher sucrose concentration.

The incubation mixture contained, per ml, 0.7 ml of mitochondrial supernatant or 0.6 ml of cell sap combined with 0.1 or 0.2 ml of resuspended “mitochondria,” microsomes or RNP particles, 0.08 μmoles of L-valine-C14 or L-leucine-C14, 1 μ mole of ATP, 10 μ moles of PEP, and, in most cases, 20 μg. of pyruvate kinase. With pyruvate kinase a slightly higher incorporation was obtained than with endogenous enzymes as the sole source of kinases for ATP generation. The potassium concentration of the incubations was 0.1 m, except when otherwise indicated. Since pyruvate kinase has a special requirement for potassium ions (9), carbamyl phosphate (15 μmoles) and carbamate kinase (25 μg) were used as an ATP-generating system in some experiments. Besides a slight change in the potassium optimum of the system (26), there was no essential difference between the results obtained under these two conditions. The incubation was stopped by the addition of TCA to a final concentration of 5 per cent. In the experiments with recombined cell sap and particular fractions, the suspensions were adjusted to equal protein contents after incubation in order to avoid differences in isotope dilution (24).

In several experiments the distribution of protein-bound radioactivity in the system was studied after further fractionation. The incubated suspensions were rapidly cooled with ice, diluted with ice-cold potassium-free medium, and fractionated into microsomes and soluble fraction by centrifugation at 105,000 × g for 60 min. In some experiments the 105,000 × g supernatant was adjusted to pH 5.1 and the precipitate separated from the supernatant by 10 minutes’ centrifugation at 10,000 × g. In these experiments the microsomes were further fractionated into RNP particles and a DOC-soluble fraction, by addition of DOC to a final concentration of 1 per cent, followed by centrifugation at 105,000 × g for 60 min. Before the addition of TCA, samples were taken from all fractions for determination of total protein contents.

Analytical methods.—Protein was determined according to Lowry et al. (33).

In mitochondria-free homogenates, RNA was determined by the method of Ceriotti (13). In suspensions of microsomes and RNP particles, the Ogur and Rosen method (43) was used. The initial extractions with cold perchloric acid were, however, omitted, since these particles contained relatively small amounts of acid-soluble nucleotides. Glycogen was determined according to Kemp and Kits van Heijningen (38). Potassium was determined by flame spectrophotometry.

For radioactivity determinations the protein precipitates were repeatedly extracted with hot TCA, ethanol, and ether, as previously described (24). The dry protein powders were plated on aluminum planchets, and the radioactivity was measured by use of a thin mica end-window Geiger-Müller tube and scaler (Tracerlab). Specific activities are given as counts/min/mg protein at infinite thinness.

RESULTS

General characteristics of microsomal stimulation.—As was mentioned in the introduction, treatment of rats in vivo with a single dose of AF or AN exerts an inhibitory influence on amino acid incorporation by isolated liver microsomes (3).2 This effect was most pronounced ca. 4 hours after the administration of the amines. A few hours later, however, the picture was distinctly different. Chart 1 illustrates an experiment in which mitochondrial supernatants were prepared in parallel from normal rats and from rats treated with AF for different periods. The incorporation capacity of these preparations was then determined under standard conditions. Even after 8 hours the amino acid incorporation by the liver preparations showed a significant enhancement. This increase reached its culmination about 20 hours after the AF administration, when it often amounted to 200 per cent or more. The height of the peak, however, showed considerable variation, partially of a seasonal nature. The stimulatory effect was obtained independently whether the AF dose was administered partially by intraperitoneal injection or entirely by stomach tube. In the latter case the effect was somewhat smaller, but this decrease could be largely overcome by dissolving AF in propylene glycol, and to some extent also by substituting AAF for AF in the serum suspension.

As Chart 2 indicates, the AF-induced stimulation showed a steady increase with increasing doses of administered amine. Doses above 2.5 mmole/kg were not tried, in order to avoid the acute toxic effects of the drug (53). In fact, the predisposition...
to such effects manifested itself even at the above dose level as a transient lameness in the legs during the first few hours after administration.

After 20 hours' treatment with AF (2.5 mmoles/kg) the RNA/protein ratio of the mitochondrial supernatants was somewhat above the normal (Table 1). It should be emphasized, however, that this increase was far from proportional to the very marked enhancement of the amino acid incorporation activity.

Chart 3 illustrates an experiment in which increasing concentrations of liver microsomes from AF-treated or normal rats were added to the incorporation system in combination with normal cell sap. When compared on the basis of equal amounts of either microsomal protein or microsomal RNA, the microsomes from the AF-treated rats gave a markedly higher incorporation than did the normal microsomes. It was of particular interest that, also at 48 hours, when the over-all effect was declining (Chart 1), the microsome stimulation still manifested itself whether protein or RNA was used as the reference (Table 2). In this respect the stimulation effect described here, even at this late stage, differed from the stimulation observed in regenerating liver, where the increased microsomal activity is more in proportion to the RNA content of the microsomes (14).

With our preparation methods, a part of the heavier microsomes sedimented with the mitochondrial fraction. The possibility was considered that these particles might have an especially high incorporation activity and that the stimulation effect described above could be due to a higher proportion of these particles remaining in the mitochondrial supernatant from treated animals. As is shown by Table 3, however, the amino acid incorporation activity of the crude mitochondrial fraction also showed an increase, parallel to that obtained with isolated microsomes, but smaller.

In the experiment shown in Chart 3, RNP particles were prepared from the same homogenates as the microsomes. On the basis of equal RNA content

<table>
<thead>
<tr>
<th>Incorporation capacity (counts/min/mg protein):</th>
<th>Control</th>
<th>AF</th>
<th>AF in per cent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA content (mg/gm protein):</td>
<td>58</td>
<td>211</td>
<td>400</td>
</tr>
</tbody>
</table>

* 2.5 mmoles/kg body wt; 20 hours.
† Incubation system as in Chart 1, but with 80 mmoles L-leucine-C¹⁴ (0.12 µc.) and a final potassium concentration of 0.074 M.
tents the stimulation effect was appreciably smaller with the isolated RNP particles than with the whole microsomes. The removal of the endoplasmic membranes from the particle preparations by the method used in these experiments obviously had a tendency to equalize the particles from treated and untreated animals.

It seemed possible that the stimulation described here could be due to a prolonged active life span of the microsomes during incubation, rather than to an increased incorporation efficiency of the system. The experiment represented in Chart 4 clearly indicates that this was not the case. The stimulation effect was very pronounced even from the beginning of the incubation period.

In cell-free liver systems from normal adult rats most of the incorporated labeled amino acid is found in the microsomal proteins and only a minor part in the proteins of the soluble fraction. In incorporation experiments with mitochondrial supernatants from AF-treated rats (Chart 5), this isotope distribution was significantly altered. Although the protein labeling increased in all fractions, the increase was relatively more pronounced in the soluble fractions. When the microsomes were further fractionated into RNP particles and a DOC-soluble subfraction, there was again a difference in that the DOC-soluble fraction showed a relatively greater increase than the particles. The stimulation effect was obviously connected with a more efficient release of labeled proteins (or proteinaceous material) from RNP particles to the other fractions.

**TABLE 3**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation† in per cent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated per mg. particle protein</td>
</tr>
<tr>
<td></td>
<td>added</td>
</tr>
<tr>
<td></td>
<td>Calculated per 200 µg. particle</td>
</tr>
<tr>
<td></td>
<td>RNA added</td>
</tr>
<tr>
<td>Microsomes</td>
<td>200</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>175</td>
</tr>
</tbody>
</table>

* 2.5 mmoles/kg body wt; 20 hours.
† Incubation: Each tube contained 0.6 ml. of cell sap and 0.1 or 0.2 ml. of particle suspension. Otherwise as in Table 1.

Experiments with cell sap from AF-treated rats.—Cell sap preparations from normal and AF-treated rats were incubated at different concentrations in combination with normal microsomes purified by gradient centrifugation. In Chart 6 the isotope incorporation values are plotted against the amounts of cell sap protein added. It appears that cell sap from the AF-treated animals gave a considerably higher incorporation than normal cell sap. This effect was not as pronounced, however, as the microsomal stimulation shown in Chart 3. The dif-
ference between the two kinds of cell sap could also be observed when isolated RNP particles were substituted for microsomes.

Chart 7 shows an incorporation experiment in which cell sap and microsomes from AF-treated and untreated animals were incubated in different combinations. After refractionation of the incubation mixtures, the distribution of labeled protein between microsomes and cell sap was determined. In comparison with normal cell sap, the cell sap from AF-treated animals not only enhanced the amino acid incorporation by both kinds of microsomes but also increased the proportion of labeled protein recovered in the soluble fraction.

It has been demonstrated (23, 26) that the amino acid incorporation by cell-free rat liver systems shows a fairly distinct potassium optimum and that the proportion of labeled protein in the soluble fractions is markedly dependent on the potassium concentration in the medium. It could, therefore, be argued that the AF-induced effects described here could be due to a modification of the potassium dependency curve. Table 4 shows, however, that the effects were obtained both at suboptimal and at optimal potassium concentrations; neither was there any significant difference in the potassium content of the two kinds of homogenates.

Chart 4.—Incorporation of L-valine-C14 into protein by mitochondrial supernatants from rat liver after AF treatment in vivo (3.5 mmoles/kg body wt; 20 hours). Incorporation capacity after varied periods of preincubation at 30°C. Each tube contained 0.7 ml. mitochondrial supernatant. After 0, 10, 20, 30, 40, and 50 min. of preincubation, 1.0 μmole ATP, 10 μmoles PEP, 35 μg. pyruvate kinase and 77 μmoles L-valine-C14 (0.5 μc.) were added to different tubes; TCA was added 9 min. later. Final volumes: 1.0 ml. Symbols indicate middle of incubation period. Open symbols: AF-treated animals; filled symbols: control animals.

Chart 5.—Incorporation of L-valine-C14 into protein by mitochondrial supernatants from rat liver after AF treatment in vivo (2.5 mmoles/kg body wt; 20 hours). Distribution of protein-bound isotope among different fractions of incubation mixture. Incubation system as in Chart 1, but at a tenfold scale. Unfract, mitochondrial supernatants; Micr, microsomes; DOC, DOC-soluble part of microsomal fraction; RNP, RNP-particles; Sup, 105,000 X g supernatant.

Specificity of stimulation.—The stimulation effect showed a certain correlation with the carcinogenic potency of the aromatic amines administered. The well known bladder carcinogen, AN, is metabolized by rat liver (7) and is, to a certain de-
gree, carcinogenic also in this organ (11). In vivo treatment with AN had approximately the same effect as AF treatment on the amino acid incorporation capacity of subcellular rat liver systems (Table 5).

When comparing rats and guinea pigs, the stimulatory effects of AF on the amino acid incorporation shows a species specificity paralleling that of the carcinogenic potency. Guinea pigs are refractory to the carcinogenic action of AF (2) and in comparison with rats they accordingly gave a considerably weaker stimulatory response to AF treatment. As is shown by Table 5, this difference could be readily observed in incorporation experiments both with mitochondrial supernatants and isolated liver microsomes 20 hours after the administration of AF (2.5 mmoles/kg). With isolated RNP particles from guinea pig liver, no stimulation whatever of the incorporation capacity after AF treatment was observed.

**Hormone dependency of AF stimulation.**—The experiments with cell-free liver systems described here were routinely carried out with animals which had been maintained without food for 16–20 hours. During this short period of starvation the glycogen content of the liver is strongly depressed. This is of practical advantage in preparing microsomes and RNP particles. In rats treated with AF, the glycogen level was several times higher than in normal, starved rats, but never reached the level of normal, fed animals (Table 6). A similar increase in the glycogen content of rat liver has been observed by Kay and Entenman (27) after whole-body x-radiation. The analogy between AF treatment and x-radiation is further illustrated by Table 7 which shows that the capability of mitochondrial supernatants of rat liver to incorporate labeled amino acids into protein was increased 20 hours after x-radiation.

According to Mole (39) the effect of x-radiation on the glycogen content of rat liver is due to an increased sensitivity to the action of glucocorticoid hormones. To gather some information about whether a similar interpretation could also be ap-
## TABLE 4
INCORPORATION OF L-VALINE-C\(^{14}\) INTO PROTEIN BY MITOCHONDRIAL SUPERNATANTS FROM RAT LIVER AT DIFFERENT POTASSIUM CONCENTRATIONS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Counts/min/mg protein</th>
<th>AF in per cent of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.11 M K(^+)</td>
<td>0.074 M K(^+)</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>Control</td>
</tr>
<tr>
<td>Mitochondrial supernatant†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>105,000 Xg supernatant</td>
<td>137</td>
<td>82</td>
</tr>
<tr>
<td>Microsomes</td>
<td>344</td>
<td>294</td>
</tr>
<tr>
<td>Supernatant/microsomes</td>
<td>0.169</td>
<td>0.065</td>
</tr>
</tbody>
</table>

\(\uparrow \) 2.5 mmoles/kg body wt; 20 hours.
† Incubation as in Chart 5 but with final potassium concentrations as indicated.

## TABLE 5
INCORPORATION OF L-LEUCINE-C\(^{14}\) INTO PROTEIN BY CELL-FREE LIVER SYSTEMS FROM RATS OR GUINEA PIGS TREATED WITH AF\(^+\) OR AN\(^+\) IN VIVO

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation † in per cent of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td>AF</td>
</tr>
<tr>
<td>Mitochondrial supernatant</td>
<td>400</td>
</tr>
<tr>
<td>Microsomes+cell sap†</td>
<td>242</td>
</tr>
<tr>
<td>RNP particles+cell sap†</td>
<td>198</td>
</tr>
</tbody>
</table>

\(\uparrow \) 2.5 mmoles/kg body wt; 20 hours.
† Incubation system as in Chart 3, but with L-leucine-C\(^{14}\) and a potassium concentration of 0.074 M. No extra pyruvate kinase added.
† Primary values calculated as total counts/min/200 \(\mu\)g added particulate RNA.

## TABLE 6
EFFECT OF AF, X-RADIATION OR CELITE ON THE GLYCOGEN CONTENT OF RAT LIVER\(^+\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glycogen content†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>91</td>
</tr>
<tr>
<td>Starved 20 hours</td>
<td>5.8</td>
</tr>
<tr>
<td>Starved 45 hours</td>
<td>4.3</td>
</tr>
<tr>
<td>Starved 45 hours; AF treatment</td>
<td>33</td>
</tr>
<tr>
<td>Starved 45 hours; x-radiation</td>
<td>61</td>
</tr>
<tr>
<td>Starved 24 hours; Celite treatment</td>
<td>19</td>
</tr>
</tbody>
</table>

\(\uparrow \) AF: 2.5 mmoles/kg body wt; x-radiation: 2,500 r (all-body irradiation); Celite: 225 mg/kg body weight, intraperitoneally. Animals were sacrificed 20 hours after treatment.
† Mg/gm dry liver weight.

## TABLE 7
INCORPORATION CAPACITY AND RNA CONTENT OF MITOCHONDRIAL SUPERNATANTS FROM RAT LIVER AFTER X-RADIATION OR TREATMENT WITH \(\Delta^1\)-HYDROCORTISONE OR CELITE IN VIVO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>L-leucine-C(^{14}) incorporation †</th>
<th>RNA content †</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-radiation</td>
<td>292</td>
<td>110</td>
</tr>
<tr>
<td>(\Delta^1)-hydrocortisone</td>
<td>455</td>
<td>120</td>
</tr>
<tr>
<td>Celite</td>
<td>229</td>
<td></td>
</tr>
</tbody>
</table>

\(\uparrow \) X-radiation: \(\geq 2500 \text{ r (whole-body irradiation)}\); \(\Delta^1\)-hydrocortisone: 100 mg/kg body wt; Celite: 225 mg/kg body wt. Animals were sacrificed 20 hours after treatment.
† Primary values calculated as counts/min/mg protein.
† Primary values calculated as mg RNA/gm protein.

## TABLE 8
INCORPORATION CAPACITY OF MITOCHONDRIAL SUPERNATANTS FROM ADRENAL-ECTOMIZED AF-TREATED RATS\(^+\)

<table>
<thead>
<tr>
<th>Dose (mmoles/kg body wt)</th>
<th>Time of AF treatment (hours)</th>
<th>Incorporation capacity in per cent of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>76†</td>
</tr>
<tr>
<td>0.5</td>
<td>21</td>
<td>88‡</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>70‡</td>
</tr>
</tbody>
</table>

\(\uparrow \) Adrenalectomy performed 24 hours prior to AF administration. Control animals adrenalectomized at the same time as corresponding AF-treated animals. Incubation as in Table 1.
† Incorporation system with L-valine-C\(^{14}\).
‡ Incorporation system with L-leucine-C\(^{14}\).

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plied to the effect of AF treatment, rats were adrenalectomized 24-40 hours prior to AF administration. As would be expected ([3], cf. “Introduction”) the incorporation capacity of cell-free liver preparations from AF-treated animals was significantly reduced 4 hours after the injection of the amine (Table 8). In the adrenalectomized animals, however, this early inhibition was never followed by a secondary stimulation of the incorporation activity. On the contrary, the inhibition persisted unmasked even after 20 hours (Table 8). It should be noted that adrenalectomy as such did not inhibit the incorporation activity of subcellular liver preparations under these conditions (cf. 30). There was rather a weak stimulation. It may be mentioned that this stimulation was not associated with any significant modification in isotope distribution between the particulate and soluble fractions.

An increased activity of subcellular rat liver preparations in amino acid incorporation, similar to that obtained by treatment with AF, AN, or x-radiation was observed after a single intraperitoneal injection of Δ1-hydrocortisone (Table 7). The treatment with high doses of this or other glucocorticoid hormones was accompanied by a relative increase in the labeling of the soluble fractions similar to that obtained by AF treatment.

It has been shown by Kenney et al. (29) that the induction of certain liver enzymes may involve a potentiation of the action of corticosteroids. A similar potentiation could be obtained by intraperitoneal injection of diatomaceous earth (Celite). As is shown by Tables 6 and 7, Celite injection evoked an increased amino acid incorporation accompanied by an increased liver glycogen deposition, similar to the effects obtained with corticosteroids.

DISCUSSION

When rats are treated with a single, large dose of AF or AN, the activity of isolated liver microsomes in amino acid incorporation is modified in a characteristic way. After a period of more or less reduced incorporation ability (5), the activity of the microsomes shows a gradual and often drastic increase. The mechanism behind the increase is very little understood at present. It was of considerable interest, however, to find that no secondary stimulation of this kind was elicited in adrenalectomized rats. In these animals, on the contrary, the primary inhibition manifested itself unmasked for at least 20 hours (Table 8).

Effects similar to those produced by the carcinogenic amines were caused by x-radiation (Table 7). Some evidence has been presented by Mole (39) suggesting that x-radiation increases the susceptibility of the tissues to glucocorticoid hormones (cf., however [51]). A similar explanation of the effects described here seems very attractive, but on the basis of the present data it seems not possible to exclude the alternative possibility that the administered amines directly or indirectly increase the secretory activity of the adrenal cortex. As is shown by Table 7 stimulation effects similar to those produced by AF were actually obtained by the administration of high doses of certain kinds of corticosteroids (5, 25, 30). It seems very doubtful, however, whether the high corticoid activities needed for producing these effects can ever be obtained in the body in connection with physiological response mechanisms. We are under the impression that AF in some way potentiates the action of the adrenal hormones in the liver cells (cf. 29).

Which alterations of the liver microsomes are responsible for the increased incorporation activity? It should be noted that the increase in incorporation activity was considerably more pronounced than the increase in RNA content (Chart 3, Table 1). This fact strongly suggests that there was no proportionate augmentation of the relative number of RNP particles in the microsomal fraction but rather a higher incorporation activity of individual particles. Three possible causes of this higher activity have been considered:

1. A modification of the properties of the microsomal membranes may influence the functions of the attached particles. That the properties of the membranes become modified under the influence of carcinogenic amines is strongly suggested by their altered structure (47) and swelling ability (1) and by the abnormally high glycogen deposition. The microsomal membranes are further the prime site of the metabolic transformation of these compounds and of their initial binding to protein (cf. “Introduction”). Experimental evidence indicating that the incorporation activity of RNP particles is in fact influenced by the microsomal membranes has previously been discussed in some detail (25). It is worth mentioning that the increase in incorporation activity after a single dose of AF culminates at approximately the same time as the accumulation of protein-bound amine (55).

2. Data obtained by Lowe et al. (32) lend themselves to the interpretation that an increasing proportion of RNP particles may become dissociated from the microsomal membranes under the influence of repeated large doses of cortisone. A dissociation of this kind may take place under the experimental conditions described here. It should be...
noted, however, that in the present experiments the relative increase in protein labeling was almost as pronounced in the detergent-soluble microsomal fraction as in the microsomal supernatant (Chart 5). Since the detergent-soluble fraction of the microsomes mainly corresponds to the membrane material, we are under the impression that the labeling of proteins in this fraction is essentially due to amino acid incorporation by membrane-associated particles. Taken together, these facts suggest that not only dissociated particles, but also particles remaining on the membranes contribute to the increased incorporation activity of the microsomal fraction as a whole.

3. The increased incorporation may be due to a higher activity of the RNP particles proper, irrespective of the structural connections of these particles. This possibility has previously been discussed (25), and it has been exemplified by the drastic activation of RNP particles which takes place in sea urchin eggs within a few minutes after fertilization (22).

Owing to the paucity of experimental data a definitive choice between these alternatives cannot be made at present. The fact that the increase was considerably more pronounced with whole microsomes than with isolated RNP particles (Chart 3) suggests, however, that the microsomal membranes or the interrelationship between membranes and particles play some role in the development or manifestation of the effect. On the other hand, some stimulation was constantly observed with RNP particles freed from membrane material, in spite of the relatively hard purification procedure to which these particles had been exposed (48). We are inclined to believe that the endoplasmic membranes are directly or indirectly involved in initiating the stimulation process but that the particles, once activated by this unknown process, may retain the higher level of activity for a certain period even after the disintegration of the cells.

The increased efficiency with which labeled protein (or protein-like material) was released from the RNP particles to the soluble fractions merits a brief comment. Increased release efficiency may be a direct consequence of an increased incorporation activity by which a relatively larger number of labeled protein molecules will reach a critical degree of completeness within the limited period of incubation. There is fairly good evidence, however, that the "release" is governed by a special enzymic mechanism partially independent of the incorporation reaction (54). It is believed that in the present experiments the increased proportion of labeled protein in the soluble fraction after AF treatment was mainly due to the first of these two factors. It should be noted, however, that an increased incorporation activity in cell-free liver systems is not necessarily accompanied by a proportionately increased release efficiency. This was, for instance, evident in the adrenalectomized rats 24 hours after the operation.

The stimulation effects discussed here may illustrate a fairly general type of secondary reaction of liver cells in response to special kinds of sufficiently intense stress (cf. 29). Within certain limits it seems to be of minor importance for the final reaction by which chemical or physical means this stress is originally imposed. As earlier mentioned a stimulation similar to that obtained by AF treatment was observed after x-radiation. An increased amino acid incorporation activity could also be obtained in response to severe mechanical irritation (Table 7). The possibility of inducing the stimulatory effects by gastric administration of AF or AAF suggests that these agents exert their stressing action at the molecular level. A conclusion of practical significance for the study of liver carcinogenesis may be drawn from the present findings. When liver preparations from animals treated with stress-inducing agents like carcinogenic aromatic amines are used in studies of protein metabolism, the possibility of interference from secondary, hormone-dependent stimulation effects must always be considered. To what extent secondary reactions of this kind contribute to the carcinogenic development during prolonged feeding of AF cannot be properly evaluated at present. Of considerable interest in this connection is, however, the observation that, as far as is known, no liver tumors are formed in hypophysectomized or adrenalectomized rats under the influence of carcinogenic aromatic amines (17, 44).

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Effects of Carcinogenic Amines on Amino Acid Incorporation by Liver Systems: I. Secondary Increase in Microsomal Activity after Aminofluorene Treatment

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