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Sterol Biosynthesis in Neoplastic Cells: Utilization of [¹⁴C]Acetate and of [2-¹⁴C]Mevalonate

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It was observed some years ago in this laboratory that mouse Ehrlich ascites-carcinoma cells failed to incorporate [¹⁴C]acetate into sterols or fatty acids and that they also failed to oxidize it to carbon dioxide. When tumour-bearing animals were fed with ¹⁴C-labelled egg-yolk lipids ([¹⁴C]cholesterol, [¹⁴C]phospholipids and [¹⁴C]triglycerides) the tumour cells 'scavenged' the lipid from the host; the specific activities of the lipids isolated from the

tumour cells and from the liver of the host were identical (G. Popják & J. Berthet, unpublished work). These observations were recently confirmed by us when tumour-bearing mice were fed with ¹⁴C-labelled lipids of *Chlorella vulgaris* (I. Y. Gore & G. Popják, unpublished work).

These findings were in agreement with those reported by others. Busch & Baltrush (1954) concluded from experiments in which [¹⁴C]acetate was

injected into tumour-bearing animals that the tumours were virtually incapable of utilizing acetate. Fish, Stokes & Hickey (1954) also concluded, from experiments with labelled acetate, that the bulk of tumour cholesterol was not synthesized in tumour tissue but was transferred from the liver. Busch (1953) had also noted that, after injection of labelled acetate into tumour-bearing animals, the bulk of radioactivity in the tumour persisted as acetate, in contrast with other tissues where 70–100 % of the radioactivity reaching the tissues was converted into non-volatile compounds over the same period of time. Medes, Thomas & Weinhouse (1953) showed, too, that the utilization of acetate for fatty acid synthesis by the neoplastic tissue studied was too slow to supply the lipid requirements of a rapidly growing tumour, and concluded that the tumour must therefore obtain its lipids preformed from the host.

The inability of tumour cells to use acetate for lipid biosynthesis is in contrast with the behaviour of embryonic tissues, which are known to convert acetate rapidly into sterol and fatty acids (Popják & Beeckmans, 1950; Popják, 1954a), and lends no support to the old theory that cancer cells revert to an embryonic type. It does suggest a possible deletion of some enzymes, or depletion of cofactors, required for lipid biosynthesis. Further, the inability to achieve a conversion as widespread in normal tissues as that of acetate into lipids may, possibly, be related to some characteristic aspect of malignancy. The invasiveness of cancer cells may be such an aspect (see review by Swann, 1958), and may be related to the lipid composition of lipoproteins of the cell surfaces.

Our interest in the problem revived after the discovery of mevalonic acid, a key intermediate in sterol biosynthesis, which is known to be synthesized from acetate. We decided to study the possible utilization of mevalonate for sterol synthesis by tumour cells to determine whether the entire chain of reactions of sterol biosynthesis was lost in these cells, and in the hope that we might obtain a demarcation of the enzymic deficiencies of sterol synthesis in tumour cells. This paper is a report on the first stage of our investigations of this complex problem.

METHODS

Buffers. 'Phosphate-KCl buffer' (0.05 M-potassium phosphate buffer, pH 7.4, containing 75 mM-KCl and 1.5 mM-MgSO₄), or 'tris-KCl buffer' (0.05 M-tris-HCl buffer, pH 7.4, containing 75 mM-KCl, 1.5 mM-MgSO₄ and 1.5 mM-potassium phosphate) were used for washing the tumour cells. Either 0.1 M-potassium phosphate buffer, pH 7.4, containing 5 mM-MgCl₂ and 30 mM-nicotinamide, or, occasionally, 0.1 M-tris-HCl buffer, pH 7.4, was used for the incubation of whole cells. For the disruption by ultra-

sonic vibration (see below) the tumour cells were suspended in the 0.1 M-phosphate buffer (containing Mg²⁺ ions and nicotinamide).

Tumours. The tumours used were either mouse Ehrlich ascites carcinoma or a rat ascites tumour derived from a benzopyrene-induced sarcoma and designated RD3. The mouse tumour was propagated by intraperitoneal inoculation of ascitic fluid and the rat tumour by the intraperitoneal injection of washed tumour cells suspended in 0.1 M-phosphate buffer, pH 7.4. The mouse tumours were harvested 8–9 days, and the rat tumours 6 days, after inoculation.

Harvesting and preparation of ascites-tumour-cell suspensions. The animals were killed by cervical fracture. The ascitic fluid was either aspirated, or allowed to drain, from an abdominal incision into ice-cold phosphate-KCl or tris-KCl buffer solution. This procedure was carried out as rapidly as possible. The mouse ascitic fluid usually contained relatively few red blood cells and most of these remained suspended after centrifuging at 180g for 5–10 min. at 0°. The supernatant was discarded. The procedure was repeated once or twice, depending on the degree of contamination with red blood cells, the tumour cells being suspended in fresh buffer and finally packed at 800g for 10 min. The rat ascitic fluid was usually grossly contaminated with red cells, which could be separated from the RD3 tumour cells only by differential lysis. After centrifuging at 180g for 15 min. at 0°, 3 vol. of water was added to the loosely packed sediment and then, after 30 sec., the osmolarity of the suspension was restored by reconstituting the original buffer by the addition of an appropriate volume of a concentrated buffer solution (either of a concentrated phosphate-KCl or tris-KCl buffer). The suspension was spun at 180g for 15 min., the supernatant was discarded, the sediment resuspended in fresh medium and packed at 800g for 5 min. The volume of the packed cells was determined and they were suspended in an equal volume either of 0.1 M-phosphate buffer, pH 7.4, containing MgCl₂ and nicotinamide, or of 0.1 M-tris buffer, pH 7.4; the nature of the buffer had no effect on the results. Such suspensions had average 'haematocrit' values of 45%, average cell counts of 35 million cells/ml. and average protein content of 110 mg./ml. The protein content was estimated by the biuret method (Gornall, Bardawill & David, 1949).

Disruption of ascites-tumour cells. Attempts to disrupt ascites-tumour cells in a Potter-Elvehjem homogenizer or by shaking with Ballotini beads in a Mickel shaker, or by lysis (Littlefield & Keller, 1957), proved unsatisfactory. A procedure was then developed with ultrasonic vibration. A Mullard ultrasonic drill, head type 7680/3, generator type L. 277, 19–21 kcy./sec. frequency [Kerry's (Ultrasonics) Limited, Romford, Essex], was used with a titanium stub. Satisfactory results were obtained when 10 ml. of a suspension of cells in buffer (1:1) was vibrated for 20 sec. in a conical centrifuge tube surrounded by ice, with the apparatus tuned to produce maximum high-pitched sound. Microscopic examination of the treated suspensions showed that at least 90 % of the cells were disrupted. The disrupted cell preparation was centrifuged at 800g for 25 min., the sediment was discarded and the turbid supernatant used in incubations; its protein content was determined by the biuret method (average value 40 mg. of protein/ml.).

Preparation of boiled extract from liver. Rat livers were homogenized as described by Cornforth, Cornforth,

Popják & Gore (1958). The supernatant obtained after centrifuging the homogenate at 105 000g for 1 hr. was boiled until no more protein coagulated (about 2 min.). The extract was filtered and the filtrate used in incubations. It was stored at -15° . Boiled extracts were also made from pig liver, obtained fresh from the slaughter house. The liver was chopped and minced in a kitchen mincer, the pulp diluted with 2 vol. of 0.1 M-phosphate buffer, pH 7.4, containing $MgCl_2$ and nicotinamide (final concentrations 5 and 30 mM respectively), and homogenized for 30 sec. in a Waring Blender. The boiled extract was prepared either by boiling the homogenate or by boiling the particle-free supernatant from centrifuging the homogenate. The filtered extracts were stored at -15° .

Coenzymes and substrates. The sources of pyridine nucleotides, ATP and glucose 6-phosphate were those given by Hele, Popják & Lauryssens (1957). DL-Methionine, pyridoxal phosphate and thiamine pyrophosphate were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Dr V. Massey kindly provided a sample of thioctic acid. DL-[2- ^{14}C]Mevalonic acid was prepared by alkaline hydrolysis at 37° of the corresponding lactone, purchased from The Radiochemical Centre, Amersham, Bucks., and was diluted with unlabelled recrystallized lactone to a specific activity of 0.2 $\mu C/\mu mole$. [carboxy- ^{14}C]Acetate was also purchased from The Radiochemical Centre.

Incubations. These were done aerobically in small conical flasks at $37-38^{\circ}$ in a Warburg bath. When carrier squalene or cholesterol was used, 10 and 1 mg./flask respectively was added after the incubation. The extrac-

tion of the unsaponifiable material and the separation of squalene and of sterol were done as described by Popják (1954b). The 3β -hydroxy sterols were precipitated as digitonides without further characterization.

Analysis of incubation products. Total unsaponifiable products were analysed by paper chromatography as described by Kodicek & Ashby (1954). Allyl pyrophosphates (3,3-dimethylallyl, geranyl and farnesyl pyrophosphate), the free allylic alcohols derived from these and the corresponding carboxylic acids, formed enzymically from mevalonate, were isolated and analysed as described by Goodman & Popják (1960) and by Christophe & Popják (1961).

Assays of ^{14}C . These were done as described by Popják (1950).

RESULTS

Comparison of the utilization of [carboxy- ^{14}C]acetate and of [2- ^{14}C]mevalonate by tumour cells in vitro

Numerous experiments were carried out both with the mouse Ehrlich ascites-carcinoma cells and with the rat RD3-ascites cells to compare the effectiveness of acetate and of mevalonate as substrates for biosynthesis of squalene, sterol and of intermediates thereof. The results obtained with the Ehrlich carcinoma and with the rat sarcoma were qualitatively very similar, and therefore only data

Table 1. *Incorporation of [^{14}C]acetate and [2- ^{14}C]mevalonate by rat ascites tumour (RD3) in vitro*

Each flask contained 5 ml. of a suspension of tumour cells prepared in tris-KCl buffer and either [carboxy- ^{14}C]acetate (5 $\mu moles$, 780 000 counts/min.) or DL-[2- ^{14}C]mevalonate (5 $\mu moles$, 161 500 counts/min.). The cell suspension contained 76 million cells/ml. (82 million cells/ml. in the original ascitic fluid), and gave a 'haematocrit' value of 40%. The flasks were incubated at 37° for 2 hr.

Incubation no. and substrate	Total unsaponifiable products			Sterol digitonides	
	mg.	^{14}C (counts/min.)	Squalene ^{14}C (counts/min.)	mg.	^{14}C (counts/min.)
1. Acetate	4.5	770	14	15.75	498
2. Acetate	5.0	690	17	15.00	454
3. Mevalonate	4.5	18 204	1 076	16.70	10 000
4. Mevalonate	4.5	18 582	868	16.50	10 720

Table 2. *Incorporation of [2- ^{14}C]mevalonate by rat ascites-tumour (RD3) cells in vitro*

Each flask contained 2 ml. of the appropriate cell preparation. The medium used was 0.1 M-phosphate buffer, pH 7.4, containing $MgCl_2$ and nicotinamide (final concentrations 5 mM and 30 mM respectively). The whole-cell suspension (1:1, v/v) had a 'haematocrit' value of 42.5% and contained 36.7 million cells/ml. Cell suspensions were disrupted ultrasonically for 40 sec.; the protein content of the disrupted cell preparations was 51.5 mg./ml. Each flask contained [2- ^{14}C]mevalonate (1 $\mu mole$, 27 936 counts/min.). The incubations were at 37° , for 2 hr., and no carrier substances were added. The data presented are for one of two similar experiments.

Tumour preparation	Total unsaponifiable products (counts/min.)	Squalene (counts/min.)	Sterol digitonides (counts/min.)	Free prenols* (counts/min.)
Whole cells	6410	563	2406	1488
Disrupted cells	3810	283	44	1711
Whole cells	5230	738	842	2356
Disrupted cells	1180	193	10	711

* Counts found in the supernatants from digitonide precipitations and shown by gas-liquid radiochromatography to correspond mainly to farnesol.

for the rat tumour are presented in the Tables. The results shown in Table 1 are typical and the contrast in the amounts of [*carboxy*- ^{14}C]acetate and of [$2\text{-}^{14}\text{C}$]mevalonate incorporated into squalene and sterols is clearly indicated.

As can be seen from Table 2, the utilization of [$2\text{-}^{14}\text{C}$]mevalonate by ultrasonically disrupted cells was less than that by whole tumour cells. The formation of total unsaponifiable products or of squalene was not abolished, but the ability to produce sterol was almost completely lost. Preparations of disrupted cells (like those of whole cells) were unable to use acetate as substrate for squalene or sterol biosynthesis, and only a few counts appeared in the total unsaponifiable products. No radioactivity could be detected in either squalene or sterols (Table 3).

Identification of free prenols (branched-chain allylic alcohols) in the total unsaponifiable products

Data in Tables 1 and 2 show that the radioactivity recovered in the total unsaponifiable products from incubations with [$2\text{-}^{14}\text{C}$]mevalonate exceeded the sum of the counts recoverable in squalene and in sterol digitonides. This situation was found to be a constant feature in our experiments, and the source of the 'excess' of radioactivity was traced to volatile substances in the supernatants from the sterol digitonide precipitations. Unfortunately, it was still not possible to balance the discrepancy in counts because, with the method of assaying radioactivity available to us at the time, some unavoidable and variable losses occurred by evaporation during the plating of the volatile samples on planchets.

Since this paper was submitted for publication, it was found (I. Y. Gore & G. Schroeffer, unpublished work) that probably most of the radioactivity unaccounted for in the total unsaponifiable extracts was associated with a number of sterol intermediates (lanosterol and others) in the biosynthesis of cholesterol that would not readily form digitonides.

The nature of the labelled material was next investigated. The total unsaponifiable products from a whole-cell incubation with [$2\text{-}^{14}\text{C}$]mevalonate were chromatographed on paper according to the method of Kodicek & Ashby (1954) and the strip was scanned for radioactivity. A peak at the origin corresponded to squalene; some radioactivity was detected in the area corresponding to cholesterol (R_f 0.29), and another peak was found corresponding to a polar substance (R_f 0.87). Gas-liquid radiochromatography (Popják, Lowe, Moore, Brown & Smith, 1959; Popják & Cornforth, 1960) of a sample of the unsaponifiable material (from which sterols had been removed as the digitonides) revealed the presence of dimethylallyl alcohol,

Table 3. *Effect of adding boiled extract of rat liver to incubations of disrupted tumour cells with [$2\text{-}^{14}\text{C}$]mevalonate and with [^{14}C]acetate*

Expt.	Tumour preparations	Substrate	Total unsaponifiable products (counts/min.)				Sterol digitonides (counts/min.)	Free prenols* (counts/min.)	Free prenols from allyl pyrophosphates	Terpenoid acids
			(counts/min.)	(counts/min.)	(counts/min.)	(counts/min.)				
A	Whole cells	[$2\text{-}^{14}\text{C}$]Mevalonate	6410	563	2406	1488	670	397		
	Disrupted cells	[$2\text{-}^{14}\text{C}$]Mevalonate	1380	149	3	589	110	97		
	Disrupted cells + boiled extract	[$2\text{-}^{14}\text{C}$]Mevalonate	6750	190	1976	778	990	2957		
B	Whole cells	[$2\text{-}^{14}\text{C}$]Mevalonate	5720	323	1011	2133	230	112		
	Disrupted cells	[$2\text{-}^{14}\text{C}$]Mevalonate	1260	161	9	722	100	59		
	Disrupted cells + boiled extract	[$2\text{-}^{14}\text{C}$]Mevalonate	5110	1507	302	1600	140	1189		
C	Disrupted cells	[<i>carboxy</i> - ^{14}C]Acetate	30	0	0	0	0	0		
	Disrupted cells + boiled extract	[<i>carboxy</i> - ^{14}C]Acetate	40	0	0	0	0	0		
D	Whole cells	[$2\text{-}^{14}\text{C}$]Mevalonate	3900	866	476	1766	140	112		
	Whole cells + boiled extract	[$2\text{-}^{14}\text{C}$]Mevalonate	4750	581	1016	1794	260	321		

* Counts found in the supernatants from digitonide precipitations and shown by gas-liquid radiochromatography to correspond mainly to farnesol.

geraniol and of farnesol, the latter being the most abundant component (over 50 %) in the mixture. These alcohols were derived, presumably, by the action of phosphatases on allyl pyrophosphates, which are known to be intermediates in squalene biosynthesis (Lynen, Eggerer, Henning & Kessel, 1958; Lynen, Agranoff, Eggerer, Henning & Mölslein, 1959; Popják, 1959; Goodman & Popják, 1960). It had been shown by Goodman & Popják (1960) and by Christophe & Popják (1961) that such liberation of allylic alcohols from their pyrophosphate esters could be achieved by a variety of phosphatases, including a phosphatase attached to microsomes.

The formation of the allyl pyrophosphates in their turn could, of course, be inferred from the fact that squalene and sterol were produced in the various preparations. The inference was verified and it was shown that after acid hydrolysis (Goodman & Popják, 1960) of the saponified incubation mixtures, free allylic alcohols could be isolated (Table 3). These were identified by gas-liquid radiochromatography, and it was found that the largest portion of the radioactivity was in nerolidol and not in farnesol. This may be taken as evidence for the synthesis of farnesyl pyrophosphate in the tumour-cell preparation, since it is known that the effect of acid on this compound is to cause not only liberation of pyrophosphate, but also an allylic rearrangement of the resulting farnesyl carbonium ion to nerolidol (Lynen *et al.* 1958; Popják, 1959; Goodman & Popják, 1960).

Factors controlling various stages in conversion of mevalonate into sterols in tumour cells

The counts in the allylic alcohols released from the pyrophosphates by acid hydrolysis were exceeded by those in the free prenols isolated from the supernatants of the digitonide precipitations (Table 3). Since it was known that the conditions of saponification used could not give rise to more than, at most, 6–10 % of free allylic alcohols from allyl pyrophosphates (Goodman & Popják, 1960), it was assumed that the pyrophosphates were largely hydrolysed enzymically during incubation. This assumption was further supported by the isolation of labelled terpenoid acids (Table 3), shown by gas-liquid radiochromatography to contain farnesoic acid and known to arise by dehydrogenation of free allylic alcohols (Christophe & Popják, 1961). Efforts were therefore concentrated on improving the yield of allyl pyrophosphates, and two straightforward assumptions were followed up, namely (a) that the inhibition by sodium fluoride of pyrophosphatases in the cell preparations might lead to greater production of prenol pyrophosphates and hence of squalene and sterol, and (b) that a supply of cofactors might rectify a de-

ficiency in the tumour cells. However, neither the addition of sodium fluoride to inhibit pyrophosphatases, nor the supply of ATP, pyridine nucleotides, glutathione and ascorbic acid singly or combined, increased the yield of squalene or sterol produced by the tumour cells. Then an observation of Kandutsch & Russell (1960), that a homogenate of preputial-gland carcinoma could convert [^{14}C]-acetate into sterols only in the presence of a boiled extract from normal rat liver, was followed up. The authors also reported that this extract could not be replaced by CoA, ATP, pyridine nucleotides or glutathione, singly or in combination. As shown in Table 3, we obtained activation of the whole biosynthetic pathway from mevalonate to sterols when either rat or pig liver boiled extract was added to the standard incubations of disrupted cells with [$2\text{-}^{14}\text{C}$]mevalonate. No incorporation of [^{14}C]acetate into squalene, sterol or allylic intermediates could be demonstrated in the presence of the boiled extract. Typical results are shown in Table 3; in one set of experiments there was, on adding the extract to disrupted cells, a particularly large increase in the incorporation of mevalonate into squalene; in the other experiment there was a higher incorporation into sterol as well (cf. Expts. A and B, Table 3). Whether this effect was operating exclusively because of counteracting some artifact of disruption was next investigated, and whole-cell incubations with added boiled extract were set up. Here the effect of boiled extract was less dramatic, but quite definite: the amount of radioactivity isolated as sterol digitonide was double that obtained from the incubation of whole cells with [$2\text{-}^{14}\text{C}$]mevalonate (Table 3, D) without the boiled liver extract.

The activity of boiled liver extract could not be imitated by sodium fluoride, cysteine, pyridoxal phosphate, methionine, thioctic acid or thiamine pyrophosphate. Since neither Kandutsch & Russell (1960) nor we, in some previous experiments, could show any activation of tumour material by additions of ATP or pyridine nucleotides, singly or together, the problem seemed at first puzzling. The difficulty was, however, resolved when we tested the following assumptions: (a) that phosphatases [pyrophosphatases and adenosine triphosphatase(s)] present in tumour-cell preparations diminished the yield of squalene intermediates; (b) that the preparations were deficient in coenzymes; (c) that the boiled liver extracts contained an inhibitor of phosphatases and supplied some necessary cofactor(s).

Table 4 shows the results obtained when sodium fluoride was used to inhibit the phosphatases and either ATP or ATP and NADPH₂ were supplied to disrupted cell preparations incubated with [$2\text{-}^{14}\text{C}$]mevalonate. Only when sodium fluoride was

Table 4. *Replacement of boiled liver extract by sodium fluoride, adenosine triphosphate and nicotinamide-adenine dinucleotide and glucose 6-phosphate**

The disrupted cell preparation and medium used were similar to those shown in Table 2. Additions of cofactors were made as shown. Each flask contained [2-¹⁴C]mevalonate (1 μ mole, 29 456 counts/min.). Incubations were at 37° for 2 hr. Carrier cholesterol (1 mg.), squalene (10 mg.) and a mixture of prenols (10 μ l.) were added to each flask before extraction.

Additions	Total unsaponifiable products (counts/min.)	Squalene (counts/min.)	Sterol digitonides (counts/min.)	Free prenols (counts/min.)
NaF (10 μ moles/ml.)	100	0	20	0
NaF (10 μ moles/ml.), ATP (5 μ moles/ml.)	1340	14	0	570
ATP (5 μ moles/ml.), NADP (2 μ moles/ml.), glucose 6-phosphate (2 μ moles/ml.)	3760	10	0	—
ATP (5 μ moles/ml.), NADP (2 μ moles/ml.), glucose 6-phosphate (2 μ moles/ml.), NaF (10 μ moles/ml.)	2630	1210	148	210

* It was found by spectrophotometric assay that particle-free supernatants of disrupted tumour-cell preparations contained glucose 6-phosphate dehydrogenase.

present could the addition of ATP and NADPH₂ replace the boiled extract, producing enhanced incorporation of [2-¹⁴C]mevalonate into squalene and sterols. In the absence of the phosphatase inhibitor, addition of ATP presumably served to produce more prenol pyrophosphates, which were then hydrolysed by the pyrophosphatases.

Evidence for acetate activation by disrupted tumour-cell preparations

Since the tumour cells could not utilize [¹⁴C]-acetate, it was of interest to find out whether they could at least form acetyl-CoA.

The RD3 tumour cells were disrupted ultrasonically in the standard way. Incubations were set up with either the supernatant after centrifuging at 800g for 25 min. or with that from spinning at 105 000g for 1 hr. Cysteine (10 μ moles/ml.), ATP (3 μ moles/ml.), CoA (0.16 μ mole/ml.) and [carboxy-¹⁴C]acetate (3 μ moles/ml.) were added and the mixtures were incubated at 36° for 1 hr.

At the end of this time, equal volumes of salt-free hydroxylamine reagent (Beinert *et al.* 1953) and of an acetate buffer, pH 5.4 [0.1 M-acetic acid-1.1 M-sodium acetate (2:8, v/v)], were added and the mixture was incubated at room temperature for at least 1 hr., according to the procedure of Lipmann & Tuttle (1945). The hydroxamates were isolated by bringing the reaction mixture to pH 9, evaporating to dryness *in vacuo* and extracting with ethanol. The hydroxamates were chromatographed on paper in isobutyric acid-28% (w/v) ammonia soln.-water (66:3:30, by vol.) and scanned for radioactivity as described by Hele *et al.* (1957).

When all the above-mentioned cofactors were present, a peak corresponding to a substance having *R_f* 0.61-0.63 was observed. This was shown to be identical with the peak produced by marker acetohydroxamate (*R_f* 0.61). This was further

confirmed by demonstrating that this peak on the radioactivity tracing corresponded to the purple spot on the paper chromatogram, produced by spraying the strip with ferric chloride in HCl. When either ATP or CoA was omitted this substance could not be detected. When sodium fluoride (3 μ moles/ml.) was included in the incubation with all cofactors, the area of the peak corresponding to acetohydroxamate was about 1.5 times as large as that obtained when no NaF was added, suggesting that the tumour-cell preparations contain also adenosine triphosphatase(s).

DISCUSSION

In this initial stage of our work we have of necessity been working without a reference normal tissue to serve as a proper control. Although the conclusions reached could thus be only empirical, the advantages of using free tumour cells uncontaminated by normal tissues decided our choice of ascites tumour cells as the first material to be studied. In the next stage, we propose to extend our investigations to well-differentiated rat hepatomas and liver-cell carcinomas, which will be compared with normal rat-liver tissue.

Our basic premise has been that the metabolic peculiarity of neoplasms is not a deficiency of sterols, since tumours do not lack cholesterol (Fish *et al.* 1954), but a failure of sterol synthesis to operate effectively in them. We have therefore attempted to investigate some features of this failure in ascites-tumour cells.

First of all a complete block has been demonstrated between acetyl-CoA and mevalonate. Since this key intermediate, mevalonate, cannot be formed endogenously, the rest of the pathway to sterol cannot operate. This is borne out by the evidence for the 'scavenging' of preformed lipids by tumours from the hosts. The possibility of

tumours overcoming the obstacle by taking up mevalonate from the host is remote. Mevalonate is metabolically very active and is not a component of a cycle, but is irreversibly converted, finally into sterols, in normal tissues. Our observations have revealed therefore a curious situation in the tumours studied. In spite of the fact that tumour cells cannot synthesize mevalonate, they have retained all the enzymes necessary for its conversion into sterol; furthermore, a pathway for the alternative metabolism of allyl pyrophosphates (derivable from mevalonate only), and which may be involved in the physiological regulation of sterol biosynthesis, is also present in the tumour cells. The powerful action of the pyrophosphatases and the suboptimum amounts of ATP and of NADPH₂ available endogenously, demonstrated in the present study, would lead to increased irreversible hydrolysis of allyl pyrophosphates to the metabolically inert allylic alcohols (Goodman & Popják, 1960), and further to their dehydrogenation to terpenoid (including farnesoic) acids (Christophe & Popják, 1961). Since farnesoic acid has been shown to inhibit squalene and sterol synthesis (Wright & Cleland, 1957) by its action on mevalonic kinase (Levy & Popják, 1960), it has been suggested (Christophe & Popják, 1961) that this sequence of events may be involved in the physiological regulation of sterol synthesis. If the presumption that tumours have little opportunity to obtain mevalonate *in vivo* is correct, the whole complex of enzymes involved in its conversion into sterol must lie dormant once the neoplasm has lost its ability to synthesize the substrate. It is tempting to think, with reference to lipid metabolism, that the presence of these enzymes may be a vestige of the tumour's former normalcy. This alteration of metabolism apparently culminates in the neoplastic cells becoming parasitic on the host for their lipid requirements. The question arising here is whether lipid 'scavenged' by the cells finds itself at a site within the cell meant for it in the course of normal intracellular biosynthesis. Such a possible translocation of lipid components within the cell structures could, presumably, result in alteration of cell organization or function or both.

SUMMARY

1. Neither the mouse Ehrlich ascites carcinoma nor a rat ascites tumour (RD3) can use acetate for fatty acid or cholesterol synthesis, but both can convert mevalonate into squalene and sterol.

2. There is a block of sterol biosynthesis in these tumour cells between the acetate and the mevalonate stages. Evidence has been obtained that the block is located after the formation of acetyl-coenzyme A.

3. The complement of enzymes necessary for the conversion of mevalonate into sterols is present in tumour cells, but certain interfering reactions prevent these from functioning efficiently.

4. The factors adversely affecting the production of squalene and of sterol from mevalonate by the tumour cells are: (a) depletion of allyl pyrophosphates through their irreversible hydrolysis to the metabolically inert free allylic alcohols by pyrophosphatases; (b) insufficient adenosine triphosphate for optimum synthesis of allyl pyrophosphates; (c) a deficiency of reduced nicotinamide-adenine dinucleotide phosphate required absolutely in the step leading to squalene formation, and also in the cyclization of squalene to sterol.

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