We wish to thank Miss Louise Hackett for technical assistance and Dr V. C. Barry, Director of the Laboratories of the Medical Research Council of Ireland, for his interest in this work, which was supported in part by a research grant (E-2842) from the Institute of Allergy and Infectious Diseases, United States Public Health Service, and by Arthur Guinness, Son and Co. (Dublin) Ltd.

REFERENCES

Aebi, H., Asselineau, J. & Lederer, E. (1953). *Bull. Soc. Chim. biol.* **35**, 661.
Bhat, M. G., Ramakrishnan, T. & Bhat, J. V. (1959). *Canad. J. Microbiol.* **5**, 109.
Brodie, B. B., Udenfriend, S. & Coburn, A. F. (1944). *J. Pharmacol.* **80**, 114.
Dagley, S., Evans, W. C. & Ribbons, D. W. (1960). *Nature, Lond.*, **188**, 560.
Edens, C. O., Creighton, M. M. & Anderson, R. J. (1944). *J. biol. Chem.* **154**, 587.
Ekstrand, T. & Sjögren, B. (1945). *Nature, Lond.*, **156**, 476.
Evans, W. C. (1956). *Rep. Progr. Chem.* **53**, 279.
Farmer, V. C., Henderson, M. E. K. & Russell, J. D. (1959). *Biochim. biophys. Acta*, **35**, 202.
Fitzgerald, R. J. & Bernheim, F. (1947). *J. Bact.* **54**, 671.
Fitzgerald, R. J. & Bernheim, F. (1948). *Amer. Rev. Tuberc.* **58**, 210.
Fitzgerald, R. J., Bernheim, F. & Fitzgerald, D. B. (1949). *Arch. Biochem.* **20**, 83.
Gale, G. R. (1952). *J. Bact.* **83**, 273.
Goris, A. & Sebetay, S. (1946). *C.R. Acad. Sci., Paris*, **233**, 933.
Hayaishi, O., Katagiri, M. & Rothberg, S. (1957). *J. biol. Chem.* **229**, 905.
Henderson, M. E. K. (1960). *J. gen. Microbiol.* **23**, 307.
Henderson, M. E. K. (1961). *J. gen. Microbiol.* **26**, 155.
Klausmeier, R. E. & Strawinski, R. J. (1957). *Bact. Proc.* p. 18.
Lehmann, J. (1946). *Lancet*, **250**, 14.
Noll, H., Rüegg, G., Gloor, U., Ryser, G. & Isler, O. (1960). *Helv. chim. acta*, **43**, 433.
Roof, B. S., Lannon, T. J. & Turner, J. C. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 38.
Shepherd, C. J. & Villanueva, J. R. (1959). *J. gen. Microbiol.* **20**, vii.
Sistrom, W. R. & Stanier, R. Y. (1953). *J. Bact.* **66**, 404.
Sloane, N. H., Samuels, M. & Mayer, R. L. (1954). *J. biol. Chem.* **206**, 751.
Srinivasan, P. R. & Weiss, B. (1961). *Biochim. biophys. Acta*, **51**, 597.
Stendal, N. (1934). *C.R. Acad. Sci., Paris*, **198**, 400.
Suda, M., Hashimoto, K., Matsuoka, H. & Kamahora, T. (1951). *J. Biochem., Tokyo*, **38**, 289.
Suda, M. & Takeda, Y. (1950). *Med. J. Osaka Univ.* **2**, 37.
Sugiyama, S., Yano, K., Tanaka, H., Komagata, K. & Arima, K. (1958). *J. gen. app. Microbiol.* **4**, 223.
Treccani, V., Walker, N. & Wiltshire, G. H. (1954). *J. gen. Microbiol.* **11**, 341.
Wagner, W. (1951). *Biochem. Z.* **322**, 121.
Walker, N. & Evans, W. C. (1952). *Biochem. J.* **52**, xxiii.
Winder, F. G. & O'Hara, C. (1962). *Biochem. J.* **82**, 98.
Yano, K. & Arima, K. (1958). *J. gen. appl. Microbiol.* **4**, 241.

Biochem. J. (1962) **84**, 506

Lipid Metabolism in the Normal and Vitamin B_{12}-Deficient Chick Embryo

BY J. H. MOORE AND B. M. DORAN
National Institute for Research in Dairying, Shinfield, Reading, Berks.

(*Received* 14 *February* 1962)

From time to time reports have appeared in the literature (reviewed by Arnstein, 1955, 1958) suggesting that vitamin B_{12} might play some part in the metabolism of lipids by animals. Since it has been established that vitamin B_{12} functions in the synthesis of labile methyl groups from more highly oxidized one-carbon-atom compounds (Arnstein, 1958), in the utilization of the α-carbon of glycine for the synthesis of ethanolamine (Stekol, Weiss & Weiss, 1952) and in the conversion of formate into serine (Arnstein, 1958), an association between vitamin B_{12} and phospholipid synthesis is self-evident. These facts led to a study in this Laboratory of the effect of vitamin B_{12} on the lipid metabolism of the chick embryo. The chick embryo seemed to present a suitable organism in which to study this relationship in view of the intensive lipid metabolism that occurs during the last week of incubation. In addition, Ferguson Rigdon & Couch (1955) had already reported histological evidence for the occurrence of fatty livers in the vitamin B_{12}-deficient chick embryo. Preliminary experiments (Moore & Doran, 1961) did not support these findings, nor did they provide any evidence for a decreased phospholipid synthesis in the livers of vitamin B_{12}-deficient chick embryos. However,

a deficiency of vitamin B_{12} did result in marked changes in the partition of certain other lipid components of the embryonic chick liver.

EXPERIMENTAL

Embryos. Vitamin B_{12}-deficient embryos were obtained from the eggs of Light Sussex hens that had been given the all-vegetable-protein diet of Coates, Harrison & Kon (1951). The hens were kept in batteries and were artificially inseminated twice weekly with semen from Rhode Island Red cocks. In Expt. 1 the hens were depleted of vitamin B_{12} for 6 months and in Expt. 2 for 8 months, by which time the hatchability of fertile eggs had fallen to about 30%. Normal embryos were obtained from the eggs of Light Sussex hens that had been given the same diet supplemented with 27·5 μg. of vitamin B_{12}/kg. These hens were kept on wire screens in fold units and naturally mated with Rhode Island Red cocks.

Embryos were removed from the eggs after 17 days of incubation in Expt. 1 and after 15, 17 and 19 days of incubation in Expt. 2. In Expt. 1 only the livers, but in Expt. 2 the livers, extrahepatic tissues and yolk sacs, were retained for analysis and pooled as shown in Table 1, so that tissues from the embryos of each hen were distributed equally among the pooled samples.

Solvents. *n*-Hexane ('fraction from petroleum'), benzene ('pure crystallizable'), chloroform, methanol ('pure') and diethyl ether ('peroxide-free'), all obtained from May and Baker Ltd., Dagenham, were purified by redistillation or by treatment with activated alumina (Böttcher, Woodford, Boelsma-van Houte & van Gent, 1959).

Extraction of lipids. Immediately after removal from the eggs, the embryonic livers, extrahepatic tissues and yolk sacs were homogenized in a suitable blender in chloroform–methanol (2:1, v/v), the homogenate was passed through a sintered-glass filter and the residue re-treated with chloroform–methanol (2:1, v/v) until lipid extraction was complete. The combined lipid extracts from each sample were purified by washing with 0·88% KCl (Folch, Lees & Sloane-Stanley, 1957).

Fractionation of lipids. The lipids were fractionated on columns of silicic acid by an adaptation of the method of Barron & Hanahan (1958). Silicic acid (100-mesh; A.R.; Mallinckrodt Chemical Works) was activated at 110° for 12 hr. and then successively washed with diethyl ether, benzene–hexane (3:17, v/v) and hexane. The columns were prepared from a suspension of silicic acid in hexane that had been boiled briefly to remove air bubbles from the silicic acid (Böttcher *et al.* 1959). Each column consisted of 3 g. of silicic acid and measured 6 cm. × 1 cm. when packed under N_2 at a pressure of 5 lb./in.^{2} Lipid samples of 55–60 mg. in hexane were placed on the columns and the various fractions eluted as shown in Fig. 1. Under these conditions a positive pressure of 5 lb./in.^{2} gave a flow rate of about 3 ml./min. for hexane, which gradually decreased to about 1 ml./min. for the most polar solvent mixture, chloroform–methanol (1:4, v/v). With the exception of the free sterol and diglyceride components, which were always eluted together by diethyl ether–hexane (1:4, v/v), separation of the various lipid fractions on these small columns was satisfactory, as may be seen from Fig. 1. Recovery of lipid from the columns was between 98 and 100% as long as the column load was not less than that recommended by Barron & Hanahan (1958), i.e. 18–20 mg. of lipid/g. of silicic acid. Refinements of technique such as further particle grading of the silicic acid and the use of water-jacketed columns as described by Hirsch & Ahrens (1958) were unnecessary.

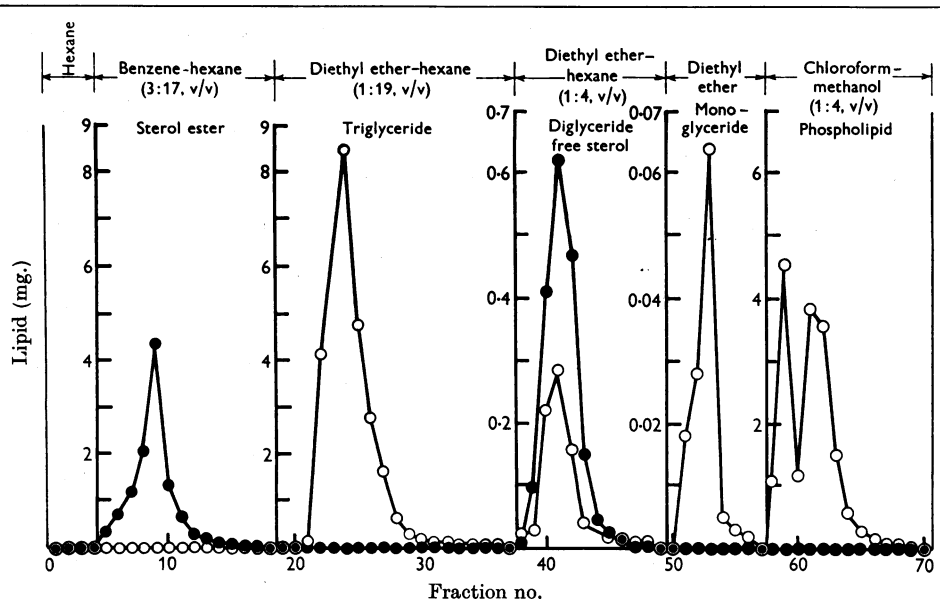

Fig. 1. Chromatography of lipids of 17-day vitamin B_{12}-deficient chick-embryo liver on a 3 g. column of silicic acid. The column eluate was collected in 4 ml. fractions. Column load: 56·3 mg. of lipid. ●, Liebermann–Burchard-reacting lipids; ○, glycerol-containing lipids.

In order to prepare phospholipid-free extracts necessary for the determination of unesterified fatty acids (Lovern, Olley & Watson, 1959), lipid samples were placed on columns of silicic acid as described above and eluted with chloroform. Under these conditions phospholipids remained on the column and the other lipids, including the unesterified fatty acids, passed into the eluate.

Methods of analysis. Evaporation of solvents was performed on a water bath at 60° under a stream of N_2. The total lipid content of the purified extracts of the various tissues was determined gravimetrically. Cholesterol was determined in the column eluates containing sterol esters on one hand and free sterol plus diglycerides on the other by an adaptation of the method of Abell, Levy, Brodie & Kendall (1952) for determining total cholesterol in serum. In view of the effect of polyunsaturated fatty acids on the Liebermann–Burchard reaction of cholesterol reported by Brown (1959) and Rhodes (1959) it is essential that the colour reaction be preceded by saponification and extraction with hexane to separate cholesterol from the fatty acids also present in these fractions.

Glyceride glycerol was determined by a modification of the method of Van Handel & Zilversmit (1957) involving saponification of the glycerides with ethanolic KOH, oxidation of the glycerol with periodate and reaction of the resulting formaldehyde with chromatropic acid. Removal of the ethanol after saponification was unnecessary as long as the liberated fatty acids and cholesterol, if present, were removed before the oxidation step by extraction of the aqueous ethanolic solution of glycerol with hexane.

Lipid phosphorus was determined by the method of Allen (1940) and unesterified fatty acids were determined in the phospholipid-free extract by the micro-titration procedure described by Albrink (1959).

Weights of cholesterol ester, unesterified fatty acid, tri-, di- and mono-glyceride were calculated from analytical results in terms of oleic acid. Weights of phospholipid were obtained by multiplying the lipid-phosphorus values by 25 (Wittcoff, 1951).

The significance of the differences between the means for the deficient and normal embryos was determined by Student's *t* test, the 1% level of significance being accepted as indicative of a real biological difference.

RESULTS

Normal embryonic tissues. Results presented in Table 1 show that the total lipid content of the normal embryonic livers increased substantially during the period of development studied. This increase in total lipid was most marked during the 17–19-day period and may be accounted for almost entirely by the very pronounced concomitant increase in esterified sterol. From 15 to 19 days of development, the triglyceride and unesterified fatty acid content of the normal embryonic livers decreased. Table 1 also shows that the results for the analysis of the 17-day-old normal embryonic livers of Expt. 1 agreed fairly well with those of Expt. 2.

As with the liver, the total lipid content of the extrahepatic tissues also increased during the 17–19-day period of incubation (Table 2), but in contrast with the livers this increase was mainly due to the triglyceride fraction. In addition, there

Table 1. *Lipid composition of the livers of vitamin B_{12}-deficient and normal chick embryos*

Mean values are given ± S.E. Results refer to Expts. 1 and 2.

	Vitamin B_{12}-deficient embryos				Normal embryos			
Age of embryos (days) ...	15	17	17	19	15	17	17	19
Expt. no.	2	1	2	2	2	1	2	2
No. of pooled groups of livers	3	6	3	4	3	3	3	2
Total no. of embryos	27	60	33	38	27	21	28	14
Component lipids (% in dry matter)								
Total lipid	28·1 ±0·80	31·7 ±1·25	33·6 ±1·01	37·5 ±0·89	25·7 ±0·52	29·0 ±1·09	29·2 ±0·52	45·1 ±1·74
Triglyceride	8·23* ±0·81	11·2* ±0·99	14·0* ±0·70	5·00* ±0·06	5·09 ±0·011	3·56 ±0·24	4·55 ±0·04	3·40 ±0·35
Diglyceride	0·486 ±0·022	—	0·502 ±0·020	0·445 ±0·008	0·460 ±0·004	—	0·430 ±0·007	0·537 ±0·026
Monoglyceride	0·0842 ±0·0025	—	0·0636 ±0·0042	0·0590 ±0·0036	0·085 ±0·0039	—	0·0587 ±0·0082	0·0766 ±0·0022
Total sterol	4·47* ±0·084	6·39* ±0·315	5·46* ±0·224	14·6* ±0·412	6·36 ±0·142	9·31 ±0·530	9·09 ±0·120	20·2 ±0·588
Sterol ester	5·46* ±0·143	8·94* ±0·500	7·38* ±1·103	22·4* ±0·72	8·71 ±0·071	13·6 ±0·593	13·3 ±0·216	31·5 ±0·90
Free sterol	1·23 ±0·016	1·09* ±0·033	1·08* ±0·017	1·32 ±0·06	1·19 ±0·072	1·22 ±0·078	1·22 ±0·01	1·15 ±0·053
Unesterified fatty acid	1·58 ±0·067	—	0·989 ±0·044	0·649 ±0·029	1·54 ±0·15	—	0·811 ±0·038	0·629 ±0·022
Phospholipid	11·4* ±0·114	9·34 ±0·280	9·56 ±0·161	7·16 ±0·28	8·64 ±0·285	9·42 ±0·284	9·03 ±0·469	7·03 ±0·273

* Significantly different ($P < 0·01$) from that in the normal embryos.

Table 2. *Lipid composition of the extrahepatic tissues of vitamin B_{12}-deficient and normal embryos*

Mean values are given ± S.E. Results refer to Expt. 2. Number of pooled groups and total number of embryos are as in Table 1.

	Vitamin B_{12}-deficient embryos			Normal embryos		
Age of embryos (days) ...	15	17	19	15	17	19
Component lipids (% in dry matter)						
Total lipid	15·1 ±0·08	18·8 ±0·08	20·3 ±0·43	15·0 ±0·36	17·9 ±0·09	20·6 ±0·05
Triglyceride	9·12 ±0·07	13·0 ±0·14	17·0 ±0·3	9·07 ±0·56	12·4 ±0·04	17·2 ±0·88
Diglyceride	0·32 ±0·032	0·465 ±0·048	0·471 ±0·023	0·348 ±0·035	0·432 ±0·042	0·531 ±0·037
Monoglyceride	0·0644 ±0·0043	0·0731 ±0·0041	0·0500 ±0·0036	0·0642 ±0·0073	0·0650 ±0·0018	0·0587 ±0·0019
Sterol ester	0·466 ±0·020	0·716 ±0·013	0·548 ±0·027	0·493 ±0·032	0·697 ±0·013	0·549 ±0·027
Free sterol	1·06 ±0·034	0·890 ±0·026	0·525 ±0·016	1·01 ±0·041	0·836 ±0·013	0·520 ±0·030
Unesterified fatty acid	0·698 ±0·022	0·645 ±0·026	0·520 ±0·013	0·656 ±0·013	0·641 ±0·020	0·545 ±0·045
Phospholipid	3·72 ±0·045	3·13 ±0·104	1·80 ±0·299	3·66 ±0·056	2·96 ±0·115	1·98 ±0·106

Table 3. *Lipid composition of the normal whole embryo*

Values are the percentages of the major components in the dry matter calculated from the mean values of Tables 1 and 2. Results refer to Expt. 2.

Age of embryo (days) ...	15	17	19
Total lipid	15·4	18·3	21·5
Triglyceride	8·93	12·1	16·7
Sterol ester	0·774	1·03	1·69
Sterol	1·02	0·848	0·557
Unesterified fatty acid	0·684	0·646	0·547
Phospholipid	3·83	3·15	2·17

was a gradual increase in diglyceride concentration but a decrease in that of free sterol, unesterified fatty acid and phospholipid.

To compare the results obtained in these experiments with earlier findings on the lipid composition of the developing embryo, the percentage of the total lipid and major lipid components in the dry matter of the normal whole embryo (i.e. livers plus extrahepatic tissues) has been calculated from the mean results presented in Tables 1 and 2 and the mean dry weights of the livers and extrahepatic tissues. These calculated values are shown in Table 3 and substantiate the previously recorded changes in the concentrations of total lipid (Murray, 1926), triglyceride (Cahn, 1928), free and esterified sterol (Tsuji, Brin & Williams, 1955), unesterified fatty acids (Kusui, 1932) and phospholipid (Kugler, 1936) that occur in the whole embryo during 15–19 days of incubation.

Unfortunately the dry-matter content of the yolk sacs was not determined, so the values for the lipid composition of these tissues are presented only as percentages of the total lipid (Table 4). As embryonic development proceeded from 15 to 19 days the proportion of unesterified fatty acid in the yolk-sac lipid tended to decrease and the proportion of free and esterified cholesterol increased. The remaining components of the yolk-sac lipid did not change substantially in proportion during this period. These findings are also in agreement with those reported from other Laboratories for concentrations of triglyceride (Budowski, Bottino & Reiser, 1961), free and esterified sterol (Tsuji *et al.* 1955), unesterified fatty acids (Kusui, 1932) and phospholipids (Kugler, 1936) in the yolk-sac lipids during embryonic development. For instance, it may be calculated from the data on the composition of the yolk sac presented by Tsuji *et al.* (1955) that the ratio esterified cholesterol: total cholesterol increased from 0·17 at 15 days to 0·28 at 18 days and to 0·39 at 21 days of development. The ratios calculated for the 15-, 17- and 19-day yolk sacs of the present experiment were 0·18, 0·28 and 0·37 respectively.

Deficient embryo tissues. The values for total lipid content of the vitamin B_{12}-deficient embryo livers were not significantly different from the corresponding values for the normal embryos, but the composition of the liver lipid of deficient and normal embryos differed very markedly (Table 1). At each stage of development the triglyceride content of the deficient liver was significantly higher and the sterol-ester content significantly lower than in the normal livers. The concentration of free sterol in the livers of the vitamin B_{12}-deficient embryos was also significantly lower than in the normal livers but only at 17 days of development. Apart from the concentration of phospholipid in the deficient embryo livers being signifi-

Table 4. *Lipid composition of the yolk sacs of vitamin B_{12}-deficient and normal chick embryos*

Mean values are given ± S.E. Results refer to Expt. 2. Number of pooled groups and total number of embryos are as in Table 1.

	Vitamin B_{12}-deficient embryos			Normal embryos		
Age of embryos (days) ...	15	17	19	15	17	19
Component lipids (% in total lipid)						
Triglyceride	72·4±0·4	71·0±0·9	69·9±0·3	73·5±0·3	72·1±0·9	71·1±0·8
Diglyceride	2·41±0·06	2·29±0·07	2·44±0·11	2·25±0·08	2·24±0·15	2·08±0·19
Monoglyceride	0·22±0·01	0·22±0·02	0·24±0·02	0·23±0·04	0·22±0·04	0·22±0·01
Sterol ester	1·12±0·02	2·37±0·04	3·88±0·02	1·11±0·02	2·44±0·02	3·92±0·03
Free sterol	3·04±0·09	3·65±0·04	3·95±0·02	2·91±0·05	3·66±0·03	3·94±0·04
Unesterified fatty acid	1·47±0·06	1·40±0·02	1·25±0·01	1·52±0·01	1·34±0·06	1·12±0·10
Phospholipid	20·5±0·20	21·0±0·51	21·0±0·18	19·8±0·10	19·3±0·80	19·2±0·05

cantly higher than in the normal ones at 15 days of incubation there was little evidence that a deficiency of vitamin B_{12} resulted in any changes in the concentration of any of the remaining lipid components. It is evident from the results presented in Table 1 that the effect of vitamin B_{12} deficiency on the liver lipids of the developing embryo is greatest at 17 days. It is also pertinent to draw attention to the fact that the accumulation of triglycerides in the deficient embryo livers at 17 days was more pronounced in Expt. 2 than in Expt. 1. This is of interest since the deficient embryo of Expt. 2 were obtained from hens that had been depleted of vitamin B_{12} for a longer period than those of Expt. 1.

Vitamin B_{12} deficiency appears to have no effect whatsoever on the lipid composition of the embryonic extrahepatic tissues (Table 2) or of the yolk sacs (Table 4).

DISCUSSION

Earlier work reviewed by Needham (1931, 1942) established that the chick embryo draws upon a succession of energy sources during development, carbohydrate metabolism predominating in the first week, protein metabolism in the second and fat metabolism in the third. Even during the last week of incubation the various lipids of the yolk are not utilized by the embryo at the same rate, for the daily increment of phospholipid in the embryo reaches a maximum at 15 days, whereas the daily increments of cholesterol and triglyceride do not reach maxima until 18 and 19 days respectively. Phospholipid molecules transported from the yolk sac to the liver are immediately degraded to products that are either utilized to synthesize new phospholipid necessary for embryonic growth or oxidized to furnish energy (Hevesy, Levi & Rebbe, 1938; Budowski *et al.* 1961). Some triglyceride transported from the yolk sac to the liver is retained or transferred to the extrahepatic tissues without degradation and resynthesis (Budowski *et al.* 1961), but the remainder is oxidatively metabolized. The accumulation of considerable quantities of esterified sterol in the liver during the last week of incubation has never been explained adequately, although Mueller (1915) considered the process of sterol-ester synthesis to be one of detoxication of the fatty acids derived from the breakdown of triglyceride and phospholipid. Stokes, Fish & Hickey (1953, 1956), who showed that the chick embryo synthesizes cholesterol from labelled acetate and that intermediates in cholesterol synthesis, e.g. cholesta-5,24-diene-3β-ol, appear in the chick embryo but are absent from the infertile egg, seem to have settled the question whether cholesterol is or is not synthesized during embryonic development. Thus the commonly observed 10% increase (Thannhauser & Schaber, 1923; Dam, 1928) in the sterol content of the whole egg during incubation must be significant.

The results obtained from the normal embryos in the present experiments are consistent with the brief account of the lipid metabolism of the chick embryo given above, but which of these processes is deranged in vitamin B_{12} deficiency is not obvious. The effects of vitamin B_{12} deficiency on the liver sterol, sterol ester and triglyceride were greater at 17 than at 15 days because of the more intensive transport of these lipids to the embryo at 17 days than 2 days earlier. Similarly, the only effect of vitamin B_{12} deficiency on the phospholipid composition of the embryonic liver was seen at 15 days, when the rate of transport of phospholipid to the embryo was maximal. The effects of vitamin B_{12} deficiency were more pronounced at 17 than at 19 days because the 17 days embryos were almost certainly more deficient than those that survived to the 19 days stage. Vitamin B_{12}-deficient hens lay eggs that vary widely in vitamin B_{12} content (Yacowitz, Miller, Norris & Heuser, 1952) and, as the maximum mortality due to vitamin B_{12} deficiency occurs at 17 days of incubation (Olcese, Couch, Quisenberry & Pearson, 1950), embryos surviving beyond this stage probably have greater reserves of the vitamin.

The effect of vitamin B_{12} deficiency on the liver lipids of the chick embryo might be due to the general retardation of development that occurs in a deficiency of this vitamin or to an interference

with the normal transport mechanism of lipids to and from the liver. Neither explanation seems valid, since vitamin B_{12} deficiency appeared to have no effect on the composition of the lipids of the yolk sacs and extrahepatic tissues in spite of the marked changes that occur in the composition of the extrahepatic tissue lipids during the 15–19 days period of development.

It would appear therefore that vitamin B_{12} deficiency in the chick embryo is associated with a decreased utilization of triglyceride and, to a smaller extent, phospholipid, a decrease in the esterification and in the synthesis of cholesterol, and that these effects are confined to the liver. It seems likely that the reduction in cholesterol-ester content of the liver is a secondary effect resulting from a decreased catabolism of triglyceride in the deficient embryo. That cholesterol synthesis is depressed in the deficient embryos is certainly indicated from the pronounced differences in the total cholesterol content of the livers, but again this effect may be secondary to the decreased triglyceride catabolism. Gould (1955) showed that the concentration of free cholesterol in the liver, rather than of total or esterified cholesterol, regulates the hepatic biosynthesis of cholesterol. Therefore in the deficient embryo liver, the reduced esterification of the free cholesterol presumably results in the inhibition of further cholesterol synthesis.

The effect of vitamin B_{12} deficiency on the lipid composition of the chick-embryo liver might thus be explained mainly in terms of a decreased utilization of triglyceride. This does not necessarily mean that vitamin B_{12} plays a direct part in fatty acid oxidation. The main source of energy available to the chick embryo is in fact triglyceride, and any decrease in the general oxidative metabolism would presumably result in its accumulation. Vitamin B_{12} deficiency has been associated with a decrease in the activity of various dehydrogenase systems (Murthy, Desikachar & Swaminathan, 1956) and of cytochrome oxidase (O'Dell, Gordon, Bruemmer & Hogan, 1955) in rat liver. None of the enzymes enumerated by Knox (1960) that specifically require glutathione as a coenzyme could play any great part in the utilization of energy by the chick embryo; but if Barron's (1951) view that a major function of glutathione in metabolism is to protect or reactivate sulphydryl enzymes is accepted, a general reduction in sulphydryl-enzyme activity might be expected in vitamin B_{12} deficiency since the latter is accompanied by lowered concentrations of glutathione. However, although the concentration of glutathione in the blood is decreased in vitamin B_{12} deficiency (Ling & Chow, 1953; Coates & Holdsworth, 1957) the position is less clear for the liver. Whereas a decrease in liver glutathione occurred in deficient rats (Register, 1954), there was no difference in the glutathione content of the livers of vitamin B_{12}-deficient and normal chicks (Coates & Holdsworth, 1957).

Any attempt to associate vitamin B_{12} with fatty acid oxidation is complicated by lack of information on the mechanism of fatty acid oxidation by the embryonic chick liver. Carey & Greville (1959) were unable to demonstrate the oxidation of octanoate, crotonate, butyrate or L-β-hydroxybutyrate by embryonic chick-liver mitochondria. They did, however, produce evidence in favour of the operation of the Krebs cycle in the mitochondria of embryonic chick liver. In the absence of any evidence to the contrary it is reasonable to assume that fatty acids are oxidized in the embryonic chick liver by a mitochondrial β-oxidation system and that the resulting acetyl-CoA units are further oxidized by entry into the Krebs cycle. This would certainly appear to be so in the chick after hatching (Carey & Greville, 1959). Nevertheless other metabolic pathways may exist and play some part in the fat metabolism of the chick embryo. It would seem that two of the steps in the β-oxidation sequence might be deranged in vitamin B_{12} deficiency: the activation of the fatty acid molecules and the thioclastic fission of the β-oxoacyl-CoA derivatives. The reports of Dubnoff (1950) that vitamin B_{12} facilitated the reduction of disulphide groups and of Boxer, Ott & Shonk (1953) that there was an accumulation of CoA in the livers of vitamin B_{12}-deficient chicks led Arnstein (1955) to postulate that the disulphide form of CoA accumulates in vitamin B_{12} deficiency and would therefore be unavailable for the activation of the fatty acids. However, Boxer, Shonk, Gilfillan, Emerson & Oginksy (1955) established that both the oxidized and reduced forms of CoA accumulate in vitamin B_{12} deficiency. It is not known whether there is a decrease in fatty acid activation in vitamin B_{12} deficiency, but Yesair (1958) has reported a lowered thiokinase activity in the livers of 21-day-old chick embryos deficient in vitamin B_{12}. He observed no decreased thiokinase activity in the livers of deficient embryos at 15 and 18 days. To explain the results of the present investigation in terms of a decreased fatty acid activation, differences in thiokinase activity should have been evident well before 21 days of development. That β-oxothiolase might be reduced in vitamin B_{12} deficiency is suggested by the work of Lynen & Ochoa (1953), which showed that this enzyme has a sulphydryl group.

There is no evidence that the rate of oxidation of any of the catalytic intermediates in the Krebs cycle is decreased in vitamin B_{12} deficiency (Boxer *et al.* 1955), but the supply of intermediates to the cycle might be decreased. Since the carbohydrate available to the chick embryo is limited in amount,

the carbon skeletons of amino acids might be an important source of Krebs-cycle intermediates in addition to glycerol. If so, a deficiency of vitamin B_{12} might result in a reduced utilization of isoleucine, valine, homoserine, homocysteine and methionine, since the catabolic pathways of these compounds involve the conversion of methylmalonyl-CoA into succinyl-CoA. The latter has been shown to be a reaction requiring vitamin B_{12} in coenzyme form by Stadtman, Overath, Eggerer & Lynen (1960).

Thus although a deficiency of vitamin B_{12} results in various metabolic derangements and certain of the derangements could account for the observed effects on the lipid metabolism of the chick embryo, this vitamin may play some role in lipid metabolism. As pointed out by Moore or Doran (1961), the analogy between the difference in the liver lipids of the normal and vitamin B_{12}-deficient chick embryo and the difference in the blood lipids of pernicious-anaemia patients before and after treatment (Williams, Erickson, Bernstein, Hummel & Macy, 1937) is striking and points to a general participation of vitamin B_{12} in lipid metabolism.

SUMMARY

1. The lipids of normal and vitamin B_{12}-deficient chick-embryo livers, extrahepatic tissues and yolk sacs after 15, 17 and 19 days of incubation have been examined by fractionation on columns of silicic acid.

2. In the normal embryos, the total lipid content of the livers increased during the last week of development and the greater part of this increase could be accounted for by the increase in esterified sterol, which accounted for 70% of the liver lipid in the 19-day-old embryos.

3. During the 17–19 days period of development the total lipid content of the extrahepatic tissues also increased but this increase was mainly due to the triglyceride fraction.

4. The only appreciable change in the composition of the yolk-sac lipids during the period of development under study was an increase in the ratio cholesterol ester:total cholesterol.

5. Although there was no difference in the total lipid content of the vitamin B_{12}-deficient and normal embryo livers, vitamin B_{12} deficiency did result in a pronounced decrease in the sterol ester and a pronounced increase in the triglyceride content at each stage of development. At 15 days the phospholipid content of the deficient embryo livers was higher and at 17 days the free sterol content was lower than in the corresponding normal embryo livers.

6. The lipids of the extrahepatic tissues and yolk sacs were not affected by a deficiency of vitamin B_{12}.

7. The differences in composition of the liver lipids are interpreted as being due mainly to a decreased utilization of triglyceride by the vitamin B_{12}-deficient chick embryo.

8. The mechanism of the effect of vitamin B_{12} deficiency on the lipid metabolism of the chick embryo is discussed.

The authors wish to thank Dr M. E. Coates, Dr S. K. Kon and Dr E. Lester Smith for valuable discussion during the progress of this work, and Miss J. Carrinci for her skilled technical assistance.

REFERENCES

Abell, L. L., Levy, B. B., Brodie, B. B. & Kendall, F. E. (1952). *J. biol. Chem.* **195**, 357.
Albrink, M. J. (1959). *J. Lipid Res.* **1**, 53.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Arnstein, H. R. V. (1955). *Symp. biochem. Soc.* **13**, 92.
Arnstein, H. R. V. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **11**, 286.
Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.
Barron, E. S. G. (1951). *Advanc. Enzymol.* **11**, 201.
Böttcher, C. J. F., Woodford, F. P., Boelsma-van Houte, E. & van Gent, C. M. (1959). *Rec. Trav. chim. Pays-Bas*, **78**, 794.
Boxer, G. E., Ott, W. H. & Shonk, C. E. (1953). *Arch. Biochem. Biophys.* **47**, 474.
Boxer, G. E., Shonk, C. E., Gilfillan, E. W., Emerson, G. A. & Oginsky, E. L. (1955). *Arch. Biochem. Biophys.* **59**, 24.
Brown, W. D. (1959). *Aust. J. exp. Biol. med.* **37**, 523.
Budowski, P., Bottino, N. R. & Reiser, R. (1961). *Arch. Biochem. Biophys.* **93**, 483.
Cahn, T. (1928). *Inaug. Diss. Paris*; cited by Needham (1931).
Carey, N. H. & Greville, G. D. (1959). *Biochem. J.* **71**, 166.
Coates, M. E., Harrison, G. F. & Kon, S. K. (1951). *Analyst*, **76**, 146.
Coates, M. E. & Holdsworth, E. S. (1957). *Rep. nat. Inst. Dairy., Reading*, p. 106.
Dam, H. (1928). *Biochem. Z.* **194**, 188.
Dubnoff, J. W. (1950). *Arch. Biochem.* **27**, 466.
Ferguson, T. M., Rigdon, R. H. & Couch, J. R. (1955). *Arch. Path.* **60**, 393.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Gould, R. G. (1955). In *Symposium on Atherosclerosis*, p. 153. Washington, D.C.: National Research Council.
Hevesy, G., Levi, H. B. & Rebbe, O. H. (1938). *Biochem. J.* **32**, 2147.
Hirsch, J. & Ahrens, E. H. (1958). *J. biol. Chem.* **233**, 311.
Knox, W. E. (1960). In *The Enzymes*, **2**, p. 253. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Kugler, O. E. (1936). *Amer. J. Physiol.* **115**, 287.
Kusui, K. (1932). *J. Biochem., Tokyo*, **15**, 319.
Ling, C. T. & Chow, B. F. (1953). *J. biol. Chem.* **202**, 445.
Lovern, J. A., Olley, J. & Watson, H. A. (1959). *J. Sci. Fd Agric.* **10**, 327.

Lynen, F. & Ochoa, S. (1953). *Biochem. biophys. Acta*, **12**, 299.
Moore, J. H. & Doran, B. M. (1961). *Biochim. biophys. Acta*, **49**, 617.
Mueller, J. (1915). *J. biol. Chem.* **21**, 26.
Murray, H. A. (1926). *J. gen. Physiol.* **9**, 405.
Murthy, V. S., Desikachar, H. S. R. & Swaminathan, M. (1956). *Nature, Lond.*, **177**, 750.
Needham. J. (1931). *Chemical Embryology*, vol. 2. Cambridge University Press.
Needham, J. (1942). *Biochemistry and Morphogenesis*. Cambridge University Press.
O'Dell, B. L., Gordon, J. S., Bruemmer, J. H. & Hogan, A. G. (1955). *J. biol. Chem.* **217**, 625.
Olcese, O., Couch, J. R., Quisenberry, J. H. & Pearson, B. P. (1950). *J. Nutr.* **41**, 423.
Register, U. D. (1954). *J. biol. Chem.* **206**, 705.
Rhodes, D. N. (1959). *Biochem. J.* **71**, 268.
Stadtman, E. R., Overath, P., Eggerer, H. & Lynen, F. (1960). *Biochem. biophys. Res. Commun.* **2**, 1.
Stekol, J. A., Weiss, S. & Weiss, K. W. (1952). *Arch. Biochem. Biophys.* **36**, 5.
Stokes, W. M., Fish, W. A. & Hickey, F. (1953). *J. biol. Chem.* **200**, 683.
Stokes, W. M., Fish, W. A. & Hickey, F. (1956). *J. biol. Chem.* **220**, 415.
Thannhauser, S. J. & Schaber, H. (1923). *Hoppe-Seyl. Z.* **127**, 278.
Tsuji, F. I., Brin, M. & Williams, H. H. (1955). *Arch. Biochem. Biophys.* **56**, 290.
Van Handel, E. & Zilversmit, D. B. (1957). *J. Lab. clin. Med.* **50**, 152.
Williams, H. H., Erickson, B. N., Bernstein, S., Hummel, F. C. & Macy, I. G. (1937). *J. biol. Chem.* **118**, 599.
Wittcoff, H. (1951). *The Phosphatides*. New York: Reinhold Publishing Corp.
Yacowitz, H., Miller, R. F., Norris, L. C. & Heuser, G. F. (1952). *Poult. Sci.* **31**, 89.
Yesair, D. W. (1958). Ph.D. Thesis: Cornell University.

Biochem. J. (1962) **84**, 513

The Surface Properties of some Neoplastic Cells

BY A. D. BANGHAM, J. C. GLOVER,
Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

SHEILA HOLLINGSHEAD AND B. A. PETHICA
Department of Chemistry, Manchester College of Science and Technology, Manchester 1

(*Received* 1 *March* 1962)

It was suggested by Bangham & Pethica (1960), as a result of electrophoresis experiments with mouse Ehrlich ascites-tumour cells, that the surface-charged groups on the tumour cells were phosphatide in character, and almost indistinguishable in this respect from the surface charges of the lymphocyte, which circulates freely in the blood (Bangham, Pethica & Seaman, 1958*b*). The phosphatide surface groups may account also for the non-adhesiveness of the tumour-cell membrane; studies of the surface properties of tumour cells are thus of interest in investigations of metastasis and malignancy.

The object of the following study has been to extend our basic knowledge of the tumour-cell surface. Investigations have been made of the effect of pH, ionic strength, and of anionic and cationic adsorption on the electrophoretic mobility of mouse Ehrlich ascites-tumour cells. Similar, though less extensive, experiments have been carried out with Klein lymphosarcoma and sarcoma 37.

EXPERIMENTAL

Preparation of cell suspensions. The neoplastic cells, mouse erythrocytes and mouse-liver cells were washed three times with 100 vol. of 0·145M-NaCl; after each washing the cells were centrifuged at 1500*g* for 3 min.

Ehrlich ascites-tumour cells. Two sources of Ehrlich ascites-tumour cells were used. Most of the results were obtained with tumour cells from C3H mice provided by Dr D. Thomason, Christie Hospital and Holt Radium Institute, Manchester (Thomason & Schofield, 1961). Cells removed from white mice, provided by Mrs Simon-Reuss, Department of Radiotherapeutics, Cambridge, gave a second source. The nature of the source is indicated in this paper by (*a*) and (*b*) respectively.

Preliminary experiments indicated that there was no variation in the mobility of the cells washed after they had been left suspended in the ascitic fluid at room temperature for 4 hr. After this period 3% of the cells were non-viable, as shown by the Nigrosine technique (Kaltenbach, Kaltenbach & Lyons, 1958). Hence the cells were left in the ascitic fluid for no longer than 4 hr. and a cell suspension was prepared for an experiment, as above, just before use.

Klein lymphosarcoma and sarcoma 37. These were obtained from Dr E. J. Ambrose, Chester Beatty Research Institute, London.

Stearic acid dispersions. These were prepared by shaking stearic acid with warm water in a stoppered all-glass tube.

Materials. All solutions were prepared in water distilled from alkaline $KMnO_4$ and redistilled from a Pyrex still. Ethidium bromide and Prothidium bromide (Watkins & Woolfe, 1956) were supplied by Boots Pure Drug Co. Ltd.; Antrycide dimethyl sulphate (Curd & Davey, 1949; Ainley, Curd, Hepworth, Murray & Vasey, 1953), Paludrine hydro-