Studies on the Properties of Factors Elevating the Activity of Mouse-Plasma Lactate Dehydrogenase

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A transmissible agent causing a 6–10-fold increase in plasma lactate-dehydrogenase activity in infected mice was described by Riley, Lilly, Huerto & Bordell (1960). This agent was present in a number of transplantable mouse tumours, and could be transmitted by the inoculation of small quantities of plasma or tissue from tumour-bearing mice into normal mice. Such infected mice could be used successively as sources of the agent, which appeared therefore to be a transmissible virus. This work has been confirmed and extended by several groups of investigators. It was shown, for example, that the virus was not present in spontaneous tumours from uninfected mice, that it was distinct from leukaemia virus, and that transplantable tumours containing the virus could be 'cured' by tissue-culture passage. From this and other evidence the agent appeared to be a 'carrier' virus picked up by mouse tumours during long periods of transplantation (Mundy & Williams, 1961; Adams, Rowson & Salaman, 1961; Yaffee, 1962; Plagemann, Gregory, Swim & Chan, 1963). Adams & Bowman (1963) found that the rate of appearance of virus elevating lactate-dehydrogenase activity in the plasma could be decreased for several hours by treating infected mice with azaserine and 6-thioguanine. During these experiments it was found that the number of infective doses of virus required to infect half the test animals (ID_{50}) rose to 10^{10}–10^{11} 24 hr. after infection. Similar results were reported by Plagemann *et al.* (1963). This high titre suggested that it might be possible to isolate the virus from infected mice in sufficient quantity to study its biochemical composition, and the present paper describes some experiments in this connexion.

MATERIALS AND METHODS

Mice. Normal adult Swiss female mice (weight 25–35 g.) were obtained from two independent sources: Simonsen Laboratories Inc., Gilroy, Calif., U.S.A., and Charles River Farms, Brookline, Mass., U.S.A.

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Plasma. Small quantities of blood (up to 0·05 ml.) were obtained from the tails, mixed with heparin (10·0 units/ml.) in 0·9% NaCl and centrifuged. Larger quantities were obtained as follows. Blood was collected from the brachial artery under ether anaesthesia, added to 10 ml. of heparin (3 units/ml.) in 0·9% NaCl in plastic 30 ml. centrifuge tubes immersed in ice until the volume in each tube reached 25 ml., and centrifuged as described below.

Source of virus. The original source was a Swiss mouse bearing a S180 ascites tumour. Plasma (0·01 ml.) in 0·1 ml. of 0·9% NaCl was inoculated intraperitoneally into normal mice, which were kept as stock infected animals, the stock being replenished by inoculating plasma from these mice into others. In experimental groups infection was produced by inoculating 0·01 ml. of plasma from an infected mouse (diluted to 0·1 ml. with 0·9% NaCl) intraperitoneally into a normal mouse. The number of infective doses of virus in such an inoculum depends on the time for which the donor mouse has been infected, ranging from about 10^8 ID_{50} 24 hr. after infection to 10^5 ID_{50} 2 weeks or more after infection. Since slightly higher virus titres seemed to be produced by the inoculation of high-titre plasma, for most of the experiments described below mice were infected with 0·01 ml. of plasma taken from mice infected with 10^5 ID_{50} 24 hr. previously.

Virus assay. The virus was assayed by inoculating serial tenfold dilutions of infected plasma into groups of three mice per dilution, and measuring the plasma lactate-dehydrogenase activity of the recipient mice 4–7 days after inoculation. The plasma lactate-dehydrogenase activity was determined as described by Adams *et al.* (1961). Activities of normal mice fell within the range 200–800 BB units/ml. of plasma, and of infected mice between about 1500 and 4000 BB units/ml. (BB units defined in Technical Bulletin no. 500, Sigma Chemical Co.).

The ID_{50} virus titre was estimated from the results of the lactate-dehydrogenase assay by the method of Reed & Muench (1938). Of necessity two or three groups of mice injected with different plasma dilutions were housed together in the same cage. According to Rowson (1963) and Plagemann *et al.* (1963), this does not result in cross-infection over the few days required for the elevation of the plasma lactate-dehydrogenase activity. We confirmed this observation in docile female mice, but found that males which show evidence of fighting cross-infect each other very rapidly. Accordingly only female mice were used for assay and were examined for evidence of skin abrasions at the relevant time.

Centrifuging. The final procedure was as follows: Blood from 100–350 mice was diluted in heparin in 0·9% NaCl as described under 'Plasma' and centrifuged in an International PR2 refrigerated centrifuge at 2° (10000*g* for

10 min.). The supernatant was removed with a Pasteur pipette, and centrifuged again at 20000*g* for 5 min. The supernatant was removed, and further diluted 2·5-fold with 0·9% NaCl, giving a final plasma dilution of about 1 in 6. The dilute plasma was then allowed to warm up to 18°, kept for 5 min., and finally centrifuged at 2° in the angle head (rotor 30) of a Spinco model L preparative ultracentrifuge (80 min. at 105000*g*). This produced a small hard-packed translucent pellet that was invisible until the tubes were emptied. The tubes were dried with filter paper, and the pellets were removed from the tubes in 0·9% NaCl, pooled and stored at −20°.

Samples of the diluted plasma before and after the centrifuging were assayed for virus elevating plasma lactate-dehydrogenase activity. The sample before centrifuging was kept at 2° until the centrifuged material was available, both being diluted and injected at the same time.

Ribonucleic acid isolation and base composition. The final procedure involved a modification of the method of Bergquist & Matthews (1962). Their procedure of ethanol precipitation, boiling with acid 70% ethanol and 95% ethanol, extracting with acetone and ether, dialysis, ethanol precipitation and removal of the ethanol by a warm air stream was followed. After the boiling with 70% ethanol the precipitate tended to become very finely divided, and required up to 20000*g* for 10 min. for complete sedimentation. After the dialysis step and removal of the ethanol, the pellet was extracted with 5 vol. of 10% (w/v) NaCl at 100° for 40 min. followed by 3 vol. for 30 min. Cold ethanol (3 vol.) was added to the combined NaCl extracts, and the mixture was left overnight at −20°. It was then centrifuged (in the International PR2 refrigerated centrifuge) at −15° for 10 min. at 2500*g*. The supernatant was rapidly decanted, the sides of the tube were dried with filter paper, and the tiny pellet was rapidly washed twice with 0·3 ml. of ethanol at 0° and finally dissolved in water.

The RNA was determined without further purification by the orcinol method (Hurlbert, Schmidt, Brumm & Potter, 1954), and in some experiments total phosphorus was also determined by the method of Robbie (1948). The base composition of the RNA was determined by making the aqueous solution 0·1 N with respect to KOH and heating it to 40° for 9 hr. Perchloric acid equivalent to the K^+ ion was then added and the solution then treated as described by Bergquist & Matthews (1962). Instead of the preliminary chromatography they describe, the final solution was made 1 N with respect to HCl and heated to 100° for 40 min. The resulting solution was evaporated to dryness *in vacuo*, taken up in 0·2 ml. of N-HCl, and run on paper for 24 hr. with adenine, guanine, cytidylic acid, and uridylic acid markers, with 70% (v/v) 2-methylpropan-2-ol made 0·8 N with respect to HCl as solvent. The spots were detected under ultraviolet light. The spots from the nucleic acid were then cut from the paper and eluted with N-HCl at 38°, and their absorption curves and concentrations determined by using a Beckman DU spectrophotometer.

Anion-exchange chromatography. Solutions containing virus were chromatographed on diethylaminoethylcellulose (DEAE-cellulose) columns (70 mm. × 10 mm.) as follows. About 1·5 g. of DEAE-cellulose was stirred with water and allowed to settle partially, half the supernatant was removed, and the procedure was repeated. The residue was placed in a 10 mm. glass column and washed with 5 ml. of N-NaOH and then with water until the eluate was neutral. The column was then used without further treatment, or treated as described below. Plasma was prepared by adding 4 ml. of blood (from four mice) to 0·1 ml. of 0·9% NaCl containing 15 units of heparin at 0° and centrifuging at 2000*g* for 10 min. (in the International PR2 refrigerated centrifuge), and the top half of the supernatant was removed (about 1 ml.). About 0·6 ml. was loaded on the column, eluted as described below and fractions (6 ml.) were collected. Virus assays were made on the fractions and on the original plasma. The extinction of each fraction was also measured at 280 mμ in a Beckman DU spectrophotometer.

RESULTS

Infectivity curves. After infection, the titre of the virus elevating plasma lactate-dehydrogenase activity rose exponentially from about 10^2 ID_{50}/ml. at 4 hr. to about 10^9–10^{11} ID_{50}/ml. at 15–25 hr., and fell to about 10^7 ID_{50}/ml. during the next 2–3 weeks. Similar results have been reported by Adams & Rowson (1961) and Plagemann *et al.* (1963). However, it soon became apparent that centrifuging of the dilute plasma for 80 min. at 105000*g* decreased the supernatant titre to about 10^7 ID_{50}/ml. over the 15 hr.–14 day period irrespective of the total titre. Fig. 1 shows total virus titre and virus titre after centrifuging at intervals after infection. Each point represents pooled plasma from six infected mice. As also shown in Fig. 1, centrifuging for 4 hr. at 105000*g* decreased the supernatant virus titre by only a little. This suggested that there were at least two different particles associated with the lactate-dehydrogenase response: one readily sedimentable, whose plasma titre rose and fell very rapidly, and the other a comparatively non-sedimentable particle whose titre rose to about 10^7–10^8 ID_{50}/ml. and remained relatively constant for at least 2 weeks.

Pellet nucleic acid. Preliminary experiments made by centrifuging undiluted plasma from in-

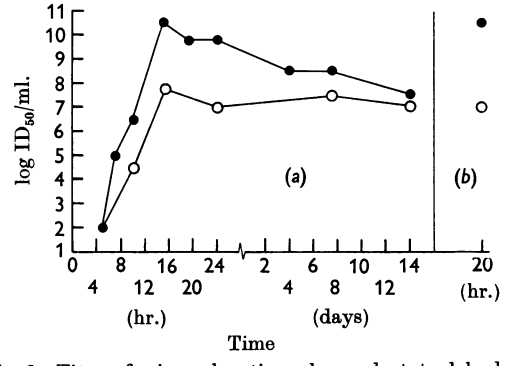

Fig. 1. Titre of virus elevating plasma lactate-dehydrogenase activity after infection of the host mice at zero-time with 10^8 ID_{50}. ●, Uncentrifuged plasma; ○, plasma centrifuged at 105000*g* for 80 min. (*a*) or 105000*g* for 4 hr. (*b*).

fected mice produced a loosely packed pellet that appeared to contain only small amounts of nucleic acid. However, dilution of the plasma as described above resulted in a hard-packed pellet from which the supernatant could easily be decanted. Attempts to isolate nucleic acid from these pellets by the classical method of Tyner, Heidelberger & LePage (1953) gave a rather bulky precipitate after precipitation of the 10% (w/v) sodium chloride extract with ethanol. Acid hydrolysis of this material and subsequent measurement of its absorption spectrum showed a peak at 265 mμ, but large amounts of fluorescent material and material absorbing below 245 mμ were also present. Attempts at paper chromatography of these hydrolysates were unsuccessful, apparently owing to an interference by these materials with the running and separation of the purine bases and pyrimidine nucleotides.

The final method adopted supports the claim of Bergquist & Matthews (1962) that their preliminary treatment substantially decreases the amount of interfering material. In four groups, a batch of 350 mice was infected, bled 16–20 hr. afterwards and the plasma (180 ml.) prepared, diluted and centrifuged as described above. Virus assays were carried out on the plasma before and after centrifuging. The pellets were pooled and nucleic acid extracted. The 10% sodium chloride extraction step was added to the method of Bergquist & Matthews (1962) for two reasons: first to assist in the characterization of the pellet nucleic acid as DNA or RNA, and secondly because it resulted in about a 30-fold purification. After alkaline hydrolysis of the ethanol-precipitated pellet and precipitation with perchloric acid a portion of the supernatant was tested for ribose by the orcinol method, and the whole of the precipitate for DNA by the diphenylamine reaction (Dische, 1955). Orcinol-positive material was found in the potassium hydroxide hydrolysate, and the sodium chloride extraction appeared to be complete since none remained in the extracted residue. No DNA was found. Total phosphorus was determined in a further portion, and the remainder used for base-ratio determination (Table 1).

Plasma from non-infected mice with normal plasma lactate-dehydrogenase activities. A number of determinations of total RNA (orcinol reaction) in pellets obtained from plasma from separate groups of 100 infected mice showed that the amounts isolated were remarkably constant at about 50–64 μg./100 ml., and bore little relationship to infective virus titre (log ID_{50}/ml., 9·5–11·0).

Accordingly plasma was prepared from 100 normal mice and centrifuged as usual. This procedure yielded a pellet similar in appearance to that obtained from the infected mice, and this was found on analysis to contain about two-thirds of the amount of RNA found in the same volume of infected plasma. Further analyses confirmed this result, and the RNA from 150 ml. of plasma was degraded and chromatographed on paper as described for that from the infected mice. The results (Table 2) showed that the plasma from normal uninfected mice (with normal plasma lactate-dehydrogenase activities) contained about 40 μg. of RNA/100 ml., and that the base ratio of the material was similar to that obtained with infected mice.

The presence of RNA in normal mouse plasma was somewhat unexpected, and raised the problem whether it was an artifact. The most likely source of material containing sedimenting RNA would seem

Table 1. *Characterization by base composition of isolated ribonucleic acid from plasma of mice infected with virus elevating plasma lactate-dehydrogenase activity*

The material was isolated from 180 ml. of plasma after infection of mice with virus. Virus assay of the plasma gave a value of 10^{11} ID_{50}/ml. The RNA content of the isolated pellet was 58 μg./100 ml. (orcinol) and the total phosphorus 4·4 μg./100 ml. The amount of base is expressed as a percentage of the total 12·1 μg. of base recovered/100 ml. of plasma. In calculating the molar ratios, adenine is taken as 1·00.

	R_F relative to corresponding marker	Amount of base (%)	Molar ratio
Adenine	1·03	22	1·00
Guanine	1·04	34·5	1·40
Cytidine	1·01*	23·5	1·25
Uridine	0·98*	20	1·15

* The pyrimidines were run as the monophosphate nucleotides.

Table 2. *Characterization by base composition of isolated ribonucleic acid from plasma of normal uninfected mice*

The material was isolated from 150 ml. of plasma from uninfected apparently healthy normal mice, with normal plasma lactate-dehydrogenase activities. The RNA content of the isolated pellet was 38 μg./100 ml. (orcinol) and the total phosphorus 3·0 μg./100 ml. The amount of base is expressed as a percentage of the total 8·4 μg. of base recovered/100 ml. of plasma. In calculating the molar ratios, adenine is taken as 1·00.

	R_F relative to corresponding marker	Amount of base (%)	Molar ratio
Adenine	1·00	23	1·00
Guanine	1·00	34·5	1·35
Cytidine	1·02*	23	1·2
Uridine	0·96*	19·5	1·1

* The pyrimidines were run as the monophosphate nucleotides.

to be tissue microsomes derived, for example, from disintegrated white cells. The diluted plasma was always warmed to 18° for 5 min. before centrifuging (see the Materials and Methods section) to decrease any microsomal contamination. However, the following experiment was carried out to obtain additional information on this point. Plasma was prepared from 200 normal mice. After the final dilution half was treated in the usual way and the remainder heated to 37° for 10 min. Both samples were centrifuged and the pellets collected. A preparation of mouse-liver microsomes was then made by homogenizing about 1·5 g. of mouse liver in 15 ml. of potassium chloride–tris buffer, pH 7·6, and centrifuging for 10 min. at 14000*g* in an International PR2 refrigerated centrifuge. A portion (5 ml.) of the supernatant was centrifuged for 90 min. at 105000*g* and the microsomal RNA determined (130 μg./ml.). A sample (0·75 ml.) of the original 14000*g* min. supernatant was added to (*a*) 200 ml. of buffer and (*b*) 200 ml. of the (dilute) plasma obtained after centrifuging without heating. The centrifuged plasma plus liver microsomes were then incubated for 10 min. at 37°. This mixture, and the microsomes in cold buffer, were then centrifuged for 90 min. at 105000*g*, and the pellets were collected and their RNA contents extracted. The results showed that heating to 37° for 10 min. made little or no difference to the amount of RNA found in the plasma pellet (22 μg. when heated, 24 μg. when not heated), but under these conditions only about 5 μg. of the 100 μg. of liver microsomal RNA added was recovered (90 μg. was recovered from cold buffer).

Since the results indicated that there was RNA-containing material circulating in normal mice, and that the amount circulating was raised by infection with virus elevating plasma lactate-dehydrogenase activity, it was decided to determine whether the circulating RNA content of plasma returned to normal as the virus titre decreased with time. Accordingly, large groups of mice were infected and their plasma RNA contents determined at intervals and compared with those of normal mice. The results appear in Table 3.

Chromatography on diethylaminoethylcellulose. Preliminary attempts were made to purify the virus by DEAE-cellulose chromatography. It was shown (Fig. 2) that the virus was almost completely retained on a DEAE-cellulose (OH^- form) column, and very little was removed by successive elution with water, 5 mM-sodium phosphate, pH 8·7, and 33 mM-sodium phosphate, pH 7. Virus could then be rapidly eluted by 33 mM-sodium phosphate containing sodium chloride (3%), pH 7. The results with plasma 20 hr. and 2 weeks after infection are compared in Fig. 2. Further experiments were made by equilibrating a DEAE-cellulose (OH^- form) column with 33 mM-sodium

Table 3. *Recovery of ribonucleic acid from mouse plasma at intervals after infection with virus elevating plasma lactate-dehydrogenase activity*

The mice used in this experiment were obtained from Charles River Farms.

Time after infection	Virus titre (log ID_{50}/ml.)	RNA recovered (μg./100 ml. of plasma)	Protein residue after extraction of RNA (mg. dry wt./100 ml. of plasma)
0 (Normal)	0	41	4·8
15 hr.	10·7	58	4·5
8 days	8·5	57	4·0
14 days	7·5	54	5·2

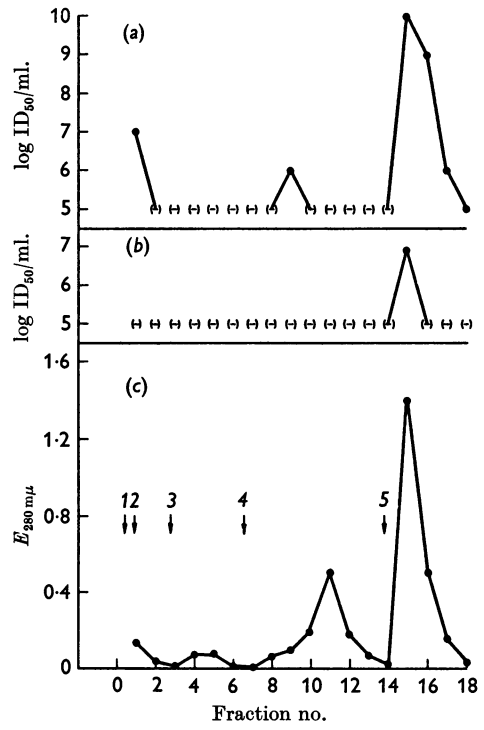

Fig. 2. Absorption of virus elevating plasma lactate-dehydrogenase activity on a DEAE-cellulose (OH^- form) column and subsequent elution. Plasma (0·6 ml.) was loaded at *1* and eluted with water (*2*), 5 mM-sodium phosphate, pH 8·7 (*3*), 33 mM-sodium phosphate, pH 7·0 (*4*), and 33 mM-sodium phosphate containing NaCl (3%) (*5*). Eighteen fractions of 6 ml. were collected and assayed for virus activity. (*a*) Virus activity in plasma from mice infected 2 weeks previously. (*b*) Virus activity in plasma from mice infected 20 hr. previously. (*c*) Extinction at 280 mμ of each plasma fraction used for virus assay. The fractions comprising (*a*) and (*b*) were separately determined and averaged to obtain the points recorded.

phosphate, pH 7, loading the plasma and eluting successively with 33 mM-sodium phosphate, pH 7, 33 mM-sodium phosphate containing sodium chloride (1%), pH 7, 33 mM-sodium phosphate containing sodium chloride (3%), pH 7, and 67 mM-sodium phosphate containing sodium chloride (3%), pH 4·4. As shown in Fig. 3 about half the virus from plasma infected 20 hr. previously came straight through the column, and most of the remainder after elution with 33 mM-sodium phosphate containing sodium chloride (1%), pH 7. However, secondary peaks were observed after elution with 33 mM-sodium phosphate containing sodium chloride (3%), pH 7, and 67 mM-sodium phosphate containing sodium chloride (3%), pH 4·4.

Plasma 2 weeks after infection gave only two major peaks of approximately equal magnitude after elution with 33 mM-sodium phosphate containing sodium chloride (1%), pH 7, and after 33 mM-sodium phosphate containing sodium chloride (3%), pH 7. A very much smaller proportion passed straight through the column than with the 20 hr. plasma.

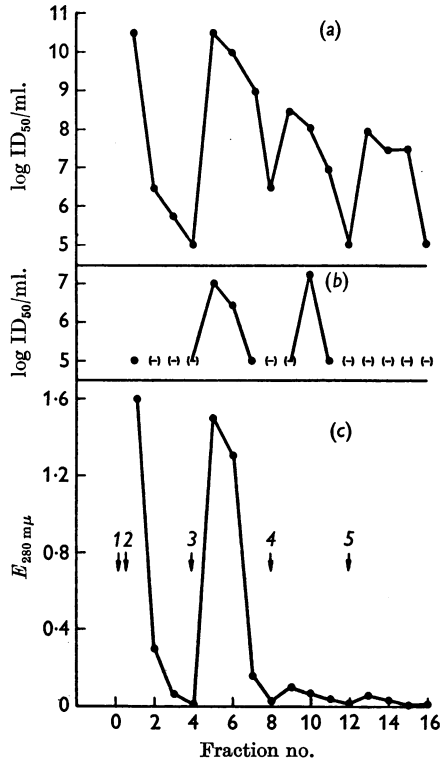

Fig. 3. As for Fig. 2, but the column was equilibrated with 33 mM-sodium phosphate buffer, pH 7·0, before loading plasma (*1*). Elution was by 33 mM-sodium phosphate, pH 7 (*2*), 33 mM-sodium phosphate containing NaCl (1%), pH 7 (*3*), 33 mM-sodium phosphate containing NaCl (3%), pH 7 (*4*), and 67 mM-sodium phosphate containing NaCl (3%), pH 4·4 (*5*).

DISCUSSION

In recent preliminary communications two apparently contradictory estimates of the particle size of virus elevating plasma lactate-dehydrogenase activity have been made: one of about 1–3 mμ (Riley, 1963) and the other of about 60 mμ (Notkins, 1963). The evidence obtained from centrifuging in the present study shows that at least two particles or groups of particles were present in infected mice, and the proportion present depended on the time after injection that the plasma assay was made. After about 15–18 hr. the readily sedimentable particle may account for 99·9% of the total, but after 2 weeks the proportions were about 1:1. The results are consistent with the proposition that the readily sedimentable particles correspond with those described by Notkins (1963) and the relatively unsedimentable particles with those of Riley (1963).

The finding of an RNA-containing ribonuclease-resistant centrifugable material in the plasma from two unrelated strains of mice was somewhat surprising. We are aware that this may be an artifact, but the evidence suggests that it is not. de Harven & Friend (1963) reported phage-like structures to be present in the blood of normal mice used as controls to animals infected with virus leukaemia.

Infection of animals with virus elevating plasma lactate-dehydrogenase activity raised the amount of plasma RNA-containing material by about 50%. The elevation was independent of the virus titre 15–20 hr. after infection, and, further, did not decrease over a 2-week period while the virus titre decreased 100-fold. The base compositions of the RNA isolated from infected and normal mice also appeared to be closely similar. Measurements made of the dry weight of the protein remaining after the extraction of RNA showed that the residual protein varied between about 4 and 9 mg./100 ml. of plasma. Some of these results are recorded in Table 3. No repeatable evidence could be obtained of an increased protein residue in infected mice, even with the highest virus titres. Obviously the pellets were contaminated to some extent with supernatant, and in some cases extraneous fibrous material (fibrin?) also appeared to be present. If it is assumed that one-third to one-half of the pellet was virus material, this gives a total of about 2 mg./100 ml. Spherical particles of 60 mμ at a concentration of 10^{11}/ml. should contribute about $1{\cdot}2 \times$ (particles/infective dose) mg./100 ml. of plasma.

It seems therefore that even if the infective dose is only one particle, the highest titres should make an appreciable addition to the total amount of recoverable material, if the normal 'virus' and the

process of infection with the virus elevating plasma lactate-dehydrogenase activity are unrelated phenomena. The evidence is in fact consistent with the suggestion that there is an association between the virus elevating plasma lactate-dehydrogenase activity and the normal virus-like material. The results obtained would be expected if the virus had taken over some of the sites of replication of the 'normal' viral material and also increased the turnover. This would exchange viral for non-viral material and increase the circulating RNA.

The results obtained by DEAE-cellulose chromatography must be regarded as preliminary, since there was no time to repeat the results with a gradient-elution technique. However, Figs. 2 and 3 showed that the virus was strongly retained on a DEAE-cellulose (OH^- form) column, and that the retentivity was greatly decreased by preliminary equilibration with 33 mM-sodium phosphate. It seems probable that the first two major peaks in Fig. 3 are due to the same material. However, the patterns of elution, with virus obtained 20 hr. after infection and 14 days after elution, were different. The results support the results from centrifuging in suggesting that there are two different particles present: in the 20-hr. plasma with a concentration ratio of about 100:1, and in the 14-day plasma of approximately 1:1.

We have no evidence for a relationship between the 'large' and 'small' viral particles, or to show why the titre of the former should decline so much more rapidly than the latter. So far, attempts to find viral antibodies have proved fruitless (Rowson, 1963) but, if there are two distinct particles present, this evidence may have to be re-examined. Possibly antibodies are produced to the large but not to the small virus. Depending on the virus–antibody reaction, there might then be considerable variation in infective titre without change in the recoverable RNA. Alternatively, if the viral material elevating plasma lactate-dehydrogenase activity has a similar protein coat to the 'normal' viral material, the titre of the former might decrease by competitive inhibition from the latter.

SUMMARY

1. The plasma of mice infected with virus elevating plasma lactate-dehydrogenase activity contains two different virus particles that can be differentiated by centrifuging and by chromatography on diethylaminoethylcellulose.

2. The larger particle, which is easily sedimentable, assays at up to 10^{11} infective doses/ml. of plasma 15–20 hr. after infection, and its titre then decreases to 10^7–10^8 infective doses/ml. 14 days after infection.

3. The smaller relatively non-sedimentable particle assays at 10^7–10^8 infective doses/ml. of plasma 15–20 hr. after infection, and its titre decreases only slightly during the next 14 days.

4. Centrifuging of infected plasma produces a pellet from which RNA (about 60 μg./100 ml. of plasma) can be isolated.

5. The amount of RNA isolated from such pellets is independent of the plasma virus titre.

6. A similar RNA-containing pellet is obtained by centrifuging plasma from apparently uninfected healthy normal mice. This pellet contains about 40 μg. of RNA/100 ml. of plasma.

7. The pellet RNA-containing material in normal plasma withstands incubation of the plasma for 10 min. at 37°. Under these conditions 95 % of added liver microsomes are destroyed.

8. The base composition of the RNA pellet from plasma with an infective virus titre of 10^{11} infective doses/ml. and of the RNA from normal plasma has been determined.

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