Table 1. Isoagglutinin Changes during Influenza Infection

Influenza A_2							
	Anti-A						
No. of two-fold dilution steps	+3	+2	+1	0	−1	−2	−3
No. of cases	0	2	31	51	19	5	0
	Anti-B						
No. of two-fold dilution steps	+3	+2	+1	0	−1	−2	−3
No. of cases	1	4	36	104	48	4	1
Influenza *B*							
	Anti-A						
No. of two-fold dilution steps	+3	+2	+1	0	−1	−2	−3
No. of cases	1	0	7	16	18	0	0
	Anti-B						
No. of two-fold dilution steps	+3	+2	+1	0	−1	−2	−3
No. of cases	1	1	10	25	23	2	0

Table 2. Distribution of ABO Blood Groups

		A	O	B	AB	Total
Influenza A_2	Observed	105	93	15	8	221
	Expected	106	88	18	9	221
Influenza *B*	Observed	28	37	7	2	74
	Expected	36	30	6	2	74

influenza infection might therefore shed light on the antigenic content of this virus.

We have followed the isoagglutinin titres in paired sera collected from 221 patients with influenza A_2 and 74 patients with influenza *B* during 1961–64. The diagnostic criteria have been clinical symptoms accompanied by a 4-fold or greater increase in influenza antibody titres (complement fixation and/or hæmagglutination inhibition tests).

Our observations are summarized in Tables 1 and 2. Sixteen paired sera showing two or more titre-step difference from first to second sample have been retitrated. By this check only 3 showed the same difference, namely a 4-fold decrease in anti-A in one case and 4-fold decreases in anti-B in two cases.

It seems therefore justified to conclude that influenza infection does not produce any change in isoagglutinin titres.

The distribution of ABO blood groups among our patients with influenza A_2 corresponds to that of a normal control population, whereas the number of influenza B patients is too small for definite conclusions.

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[1] McDonald, J. C., and Zuckerman, A. J., *Brit. Med. J.*, ii, 89 (1962).
[2] Springer, G. F., and Tritel, H., *Science*, **138**, 687 (1962).
[3] Springer, G. F., *Transfusion*, **3**, 233 (1963).

PATHOLOGY

Effects of Adenine and Guanine on Hepatic Glucose Release and on the Action of Insulin on the Liver

We have recently obtained information from work on normal subjects and mild and severe diabetics indicating that insulin has an increasing effect on hepatic glucose metabolism in untreated diabetics with progressively elevated fasting blood sugar-levels and glycosuria implying greater degrees of catabolism and gluconeogenesis[1]. There is also evidence of a hepatic action of insulin when diabetic patients are treated with phenethylbiguanide[2]. In view of the similarity between the ethylbiguanide side-chain and the nucleotides adenine and guanine, we wished to test the effect of these compounds on hepatic carbohydrate metabolism and on the action of insulin on the liver.

Using the rat liver perfusion technique developed by one of us[3,4], the release of glucose over two successive periods of 1 h has been investigated. Control levels were measured during perfusions with glucose-free tyrode solution and the effects of added adenine (in two concentrations, designated as Adenine, 0·25 γ/ml., or adenine, 0·025 γ/ml.), guanine (designated as Guanine, 0·25 γ/ml., or guanine, 0·025 γ/ml.) alone or with insulin (in two concentrations designated as Insulin, 20 milliunits/ml., or insulin, 0·2 milliunits/ml.) on hepatic glucose release expressed in mg/h have been examined so far.

Table 1

Perfusion	No. of expts.	Hepatic glucose release (mean ± *S.D.*) 1st h	2nd h
Control	12	4·7 (1·2)	4·7 (2·9)
Insulin	8	3·7 (1·0)	3·0 (1·2)
adenine	8	5·5 (1·8)	5·1 (2·1)
Adenine	10	10·4 (2·7)	8·3 (4·0)
Adenine + Insulin	8	8·3 (1·8)	2·5 (1·1)
Adenine + insulin	8	3·6 (1·2)	3·9 (1·6)
guanine	8	4·4 (2·7)	2·7 (1·8)
Guanine	10	8·3 (1·7)	8·3 (2·0)
guanine + Insulin	8	4·2 (2·6)	1·7 (0·9)
Guanine + insulin	8	4·6 (2·5)	3·1 (2·3)

The nucleotides guanine and adenine caused statistically significant increases in hepatic glucose release. Insulin, in concentrations which in previous work[3] failed to reveal a statistically significant action in controlled circumstances, suppressed the glucose-releasing action of the nucleotides, even at the lower dose-levels, suggesting that its action is hormonal rather than stoichiometric.

The results (Table 1) appear to be in close harmony with, and provide some degree of explanation for, the clinical findings here. If we consider the state of affairs in the fasting untreated diabetic, increasing demands for gluconeogenesis to offset losses through glycosuria will be associated with increasing protein catabolism. It seems probable that the concentrations of nucleotides, no longer involved in protein synthesis, might increase in the tissues, diffuse into the blood stream and perfuse the liver as well as accumulating locally from the cells in that organ. From the present evidence, it would appear that in low concentrations nucleotides may increase hepatic glucose release and that, in such circumstances, insulin, in levels which approach the physiological for the portal circulation, has a powerful action restraining hepatic glucose release.

Further work on the role of these phenomena on glucose homeostasis is being carried out.

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[1] Butterfield, W. J. H., and Whichelow, Margaret J. (submitted to *Clin. Sci.*).
[2] Butterfield, W. J. H., Fry, I. K., and Whichelow, Margaret J., *Lancet*, ii, 563 (1961).
[3] Kaldor, A., and Pogatsa, G., *Fourth Intern. Diabetes Congr.*, Geneva (1961).
[4] Kaldor, A., and Pogatsa, G., *Diabetes*, **2**, Supp. 16 (1962).

Preparation of Antigens Specific of Human Breast Carcinoma by an Immunochromatographic Method

In spite of a rapidly mounting literature on the problem of specific antigens in human neoplasms, there have been few attempts to separate these antigens in amounts permitting biochemical and immunological analyses of the same. Such analyses not only would shed light on carcinogenic mechanisms, including possibly the serological back-tracking of eventual biological agents, but would make possible a much-desired immunological classification of tumours. Work from this laboratory has been concentrated on such efforts.

In previous reports we have described methods to extract potential antigenic proteins from human tissues, both normal and neoplastic[1-3]. Even though immunodiffusion and direct cytotoxicity tests have indicated amply the existence of specific precipitinogens and cytotoxinogens in neoplastic cells, the antibodies hitherto produced on a large scale in large animals were not always totally devoid of activity against normal antigens from normal cells.

The production of large amounts of hyperimmune γ-globulins required the use of large animals and hence the need for increasing amounts of antigens. These requirements automatically limited the possibilities of inoculating separate animals with separate tumours and compelled us to work with large pools of different tumours and of normal tissues.

Departing now from antibodies produced against pooled tumour antigens and pooled normal tissue antigens, we investigated a more homogeneous pool consisting of 4 adenocarcinomas of the breast.

As reported previously[2], a major step introduced by this laboratory in the separation of tumour-specific antigens is the elimination of normal antigenic components from the tumour antigen complement by absorption with antibodies produced against a large pool of normal tissues obtained from a pool of serum from four different horses.

The dynamics of this absorption in a system of soluble antigens and soluble antibodies was previously described and all the inherent difficulties of this totally unknown system discussed. The recent introduction in our methodology of a form of insolubilized immune γ-globulin[4] greatly simplified and improved the absorption of normal components from the tumour antigen complement, free of residual horse γ-globulin.

Diazotization of horse hyperimmune anti-normal human tissue γ-globulin results in cross-linking of the globulin to an insoluble form (polyglobulin) which can be used as a chromatographic column bed[4,6]. This communication describes the use of such a column for the separation of tumour-specific antigens from breast carcinoma.

Sources of breast carcinoma: 1, 2 and 3: 60 g each of tumour tissue (adenocarcinoma of the breast, grade II) obtained from surgical specimens (mastectomies); 4: 60 g of a liver totally and diffusely infiltrated by cells of adenocarcinoma of the breast.

A total of 240 g of clean tumour tissue was homogenized in glycine buffered saline, *p*H 10·4, and fluorocarbon as described elsewhere[1] and a solution containing 3·5 g per cent of protein obtained. This will here be referred to as unabsorbed breast antigen.

Equine hyperimmune anti-tumour γ-globulin (*HTUGG*, Lot. No. 27 GG33) with a precipitin titre, in single-gel diffusion, of 2^{-5} against the inoculating antigen. This antigen was obtained by fluorocarbon treatment of a large pool of solid tumours in which breast adenocarcinomas were largely represented[2].

Equine hyperimmune anti-normal γ-globulin (*NHGG*, Lot. No. 4,5 GG21) with a precipitin titre of 2^{-7} against the inoculating antigen which was obtained by fluorocarbon treatment of a pool of nearly every normal tissue in the human body[2].

Copolymerized NHGG: Insolubilization of the anti-normal γ-globulin by cross-linkage with tetrazobenzidine bridges was obtained as described elsewhere[4].

A chromatographic column of this insoluble *NHGG* was prepared with 30 g of γ-globulin. This column was equilibrated with 0·1 M phosphate buffered saline, *p*H 7·2, at 2° C. This temperature is maintained by a jacket of crushed ice surrounding the column.

An aliquot of 180 ml. of unabsorbed breast tumour antigen containing 6·3 g of protein in glycine buffered saline *p*H 10·4 was placed over the column and allowed to soak through by removing 180 ml. of phosphate buffered saline (fraction 0). The column was then incubated for 1 h at 2° C and several fractions of 100 ml. each collected. Phosphate buffered saline was added in amounts of 100 ml. to promote descent of each new fraction.

The size of the γ-globulin column was calculated to exceed five times the weight/weight ratio of the precipitin titre previously obtained in single-gel diffusion hæmatocrit tubes[5].

Each fraction was tested in single gel diffusion for quantitative precipitation against *HTUGG* and *NHGG* and in double gel-diffusion for identification of reactants. Protein concentrations were determined by the absorption at 280 mμ wave-length.

Table 1 and Fig. 1 summarize the results obtained. In them it is apparent that: (1) The unabsorbed tumour antigen complement consists of 8 antigens, 2 of which are in common with normal tissues (Fig. 1, No. 1). (2) Antigens reacting against anti-normal γ-globulin do not appear in any of the collected fractions. (3) Increase in protein concentration results in increase in the number of tumour antigens while normal antigens remain undetected. (4) The largest number of tumour antigens (that is, 6) equals the number of antigens in the unabsorbed original

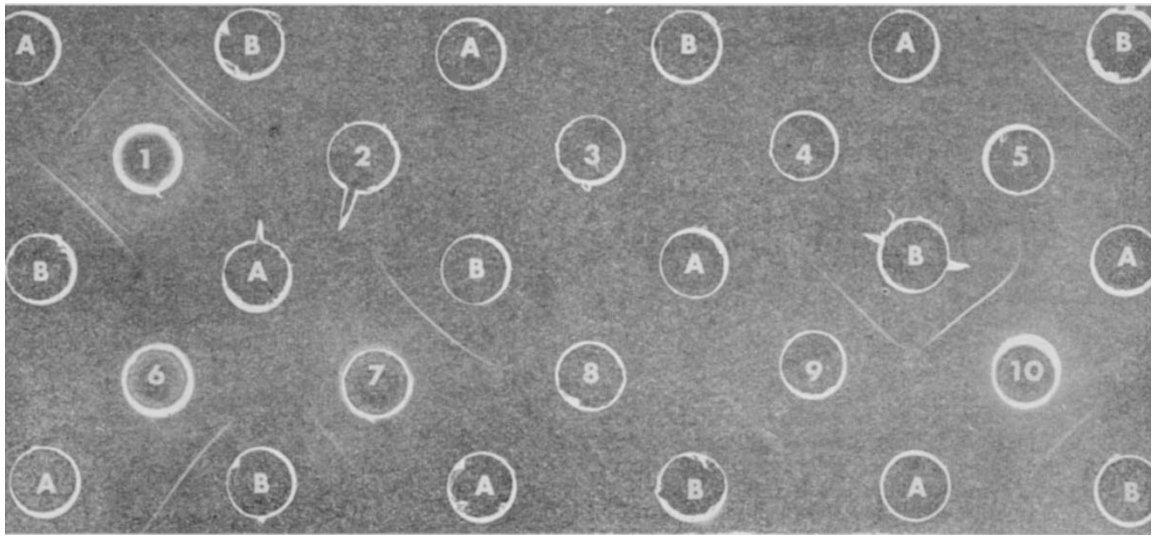

Fig. 1. Double gel-diffusion plate. *A*, equine hyperimmune anti-normal γ-globulin at 16 per cent concentration; *B*, equine hyperimmune anti-tumour γ-globulin at 16 per cent concentration. 1, unabsorbed fluorocarbon extract of breast carcinoma showing 8 lines against *B* and 2 against *A*, the last identical with 2 against *B*; 2, fraction 1 (see Table 1), only one line against *B* is shown; 3, fraction 2, only one line against *B* is shown; 4, fraction 3, a faint line which could be seen in the plate did not reproduce in the photograph; 5, fraction 4, two lines against *B*, only; 6, fraction 5, six lines may be seen against *B*, none against *A*; 7, fraction 6, at least 4 lines may be counted against *B*, none against *A*. Other numbers are not relevant to the context of this communication

Table 1. Immunochromatographic Separation of Breast Carcinoma Antigens on a Column of Cross-linked Horse Anti-normal (Human) γ-Globulin

Fraction	Titre in single gel-diffusion Against *NHGG*	Against *HTUGG*	Protein concentration g%	No. of lines in double gel diffusion (Fig. 1) Against *NHGG*	Against *HTUGG*
Unabsorbed tumour antigens	2^{-7}	2^{-5}	3·5	2	8
0 (1st 100 ml.)	0	0	0·0	0	0
1 (2nd 100 ml.)	0	2^{-3}	0·070	0	1
2 (3rd 100 ml.)	0	2^{-3}	0·062	0	1
3 (4th 100 ml.)	0	2^{-2}	0·051	0	1
4 (5th 100 ml.)	0	2^{-4}	0·480	0	2
5 (6th 100 ml.)	0	2^{-4}	1·680	0	6
6 (7th 100 ml.)	0	2^{-5}	0·600	0	4

NHGG, equine hyperimmune anti-normal γ-globulin; *HTUGG*, equine hyperimmune anti-tumour γ-globulin.

extract (that is, 8) which react with anti-tumour globulin (Fig. 1, No. 1 and *B*) minus the antigens in the same (that is, 2) reacting with antinormal globulin (Fig. 1, No. 1 and *A*). Analysis of the protein complement of breast tumour tissue reveals only antigenic acquisition but no antigenic deletion or deviation, 70 per cent of the protein being of normal antigenic nature.

These results seem to indicate that this method resulted in the resolution and large-scale preparation of specific antigens from human adenocarcinoma of the breast.

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[1] DeCarvalho, S., *J. Lab. Clin. Med.*, **56**, 333 (1960).
[2] DeCarvalho, S., *Cancer*, **16**, 306 (1963).
[3] DeCarvalho, S., Rand, H. J., and Ashby, M., *Exp. Mol. Path.*, Supp. 2 (1963).
[4] DeCarvalho, S., Lewis, A., Rand, H. J., and Uhrick, J., *Nature* (in the press).
[5] DeCarvalho, S., Uhrick, J., and Rand, H. J., *Nature*, **194**, 1275 (1962).
[6] DeCarvalho, S., Lewis, A. J., Rand, H. J., and Uhrick, J., Sixth Internat. Cong. Biochem., Abstract II–44, New York, 1964.

Effect of Early Thymectomy on Development of Mammary Tumours in Mice

It is now well established that the thymus plays an important part in the development of immunological competence in mammals. For example, in the mouse, removal of the thymus within the first 24 h after birth results in a serious impairment of the capacity to produce circulating antibody and the ability to reject allogeneic grafts of normal or neoplastic tissues. In addition, these mice also develop a wasting syndrome ending in early death[1–7]. Surgical ablation of the thymus at 6 and up to 35 days of age also produces a state of immunological impairment, which is more severe when thymectomy is performed earlier in life. These animals do not develop the wasting syndrome and have a life-span comparable with that of non-thymectomized controls[8–10].

Since development of mammary tumours in susceptible strains of mice depends at least in part on the presence of an infectious agent transmitted by the mother to the progeny via the milk (Bittner's virus), it was considered of interest to ascertain whether or not thymectomy performed at 6 days of age in mice of a high cancerous strain would affect the spontaneous development of mammary tumours in these animals.

Two groups of female mice of the *C3H/Bi* strain were used. One group of animals was thymectomized 6 days after birth by the technique routinely used in this laboratory[11], and the other left as non-thymectomized controls. After surgery, mice were raised by their own mothers and weaned at 30 days of age, at which time the thymectomized and non-thymectomized mice were housed in plastic cages in groups of 4–5 female mice per cage. One normal male of the same strain was introduced into each cage and the mice were allowed to breed. Breeding behaviour was assessed by the number of litters born from each mother in both groups (Table 1). The mice at all times had free access to 'Purina Laboratory Chow' and tap water.

The incidence of spontaneous mammary tumours in both groups was determined by weekly inspection of each individual mouse. The age of the mouse was recorded at the time a tumour appeared.

Table 1. Incidence of Spontaneous Mammary Adenocarcinoma in Thymectomized and Non-thymectomized Female Mice of the *C3H/Bi* Strain kept as Breeders

Group	No. of mice	Breeding behaviour. No. of litters born (mean)	No. of mice with cancer	%	Mean cancer age (days ± *S.D.*)
C3H normal	73	4·4	69	94·5	276 ± 5·04
C3H thymect. at 6 days of age	26	4·6	15	57·6	335 ± 16·2

The results of these experiments (Table 1) demonstrate that, in the group of *C3H* mice thymectomized at 6 days of age, 57·6 per cent of the females developed spontaneous mammary tumours as compared to 94·5 per cent in the group of non-thymectomized controls. This difference is statistically significant at the 1 per cent level. Furthermore, thymectomized mice developed tumours significantly later than non-thymectomized controls. In the former group the average cancer age was 335 ± 16·2 and in the latter 276 ± 5·04 days.

Although the interpretation of these results must remain a matter of conjecture at the present time, a few pertinent comments are in order. According to Bittner[12] development of breast cancer in mice is dependent on the concurrent effect of three factors, namely, inherited susceptibility, proper hormonal stimulation, and the presence of the mammary tumour 'agent' or virus which is transmitted from the mother to the offspring by the milk. Since the inherited susceptibility for tumour development is genetically controlled and presumably does not change after thymectomy, it might be that the mechanism operating in cancer prevention by early thymectomy, as reported herein, could be related to an alteration of the hormonal stimulation for tumour development. Another possibility is that the thymus may be essential early in life for proper growth and multiplication of the mammary tumour virus. Finally, the development of mammary tumours could be related to the immunological responsiveness of the host animal to the virus. It has been shown recently that neonatal thymectomy in mice greatly reduces the mortality resulting from inoculation with lymphocytic choriomeningitis virus (LCM), and this reduction in mortality is the result of decreasing the host response to the virus[13,14]. Perhaps the development of spontaneous mammary tumours in mice is also in part dependent on a similar mechanism.

Experiments designed to investigate these and other possibilities are in progress.

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[1] Miller, J. F. A. P., *Lancet*, ii, 748 (1961).
[2] Archer, O., and Pierce, J. C., *Fed. Proc.*, **20**, 26 (1961).
[3] Martinez, C., Kersey, J., Papermaster, B. W., and Good, R. A., *Proc. Soc. Exp. Biol. and Med.*, **109**, 193 (1962).
[4] Parrott, D. M. V., and East, J. Gu, in *The Thymus in Immunobiology*, edit. by Good, R. A., and Gabrielsen, A. (Harper and Row, New York, 1964) (in the press).
[5] Miller, J. F. A. P., *Proc. Roy. Soc.*, B, **156**, 415 (1962).
[6] Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O., Pierce, J. C., and Papermaster, B. W., *J. Exp. Med.*, **116**, 773 (1962).
[7] Miller, J. F. A. P., *Adv. Immunol.*, **2**, 111 (1962).
[8] Parrott, D. M. V., and East, J., *Nature*, **195**, 347 (1962).
[9] Hilgard, H. R., Sjodin, K., Martinez, C., and Good, R. A., *Transplantation*, 1964 (in the press).
[10] Dalmasso, A. P., Martinez, C., and Good, R. A., in *The Thymus in Immunobiology*, edit. by Good, R. A., and Gabrielsen, A. (Harper and Row, New York, 1964) (in the press).
[11] Sjodin, K., Dalmasso, A. P., Smith, J. M., and Martinez, C., *Transplantation*, **1**, 521 (1963).
[12] Bittner, J. J., *Pub. Health Rep.*, **54**, 1590 (1939).
[13] Levey, R. H., Training, N., Law, L. W., Black, P. H., and Rowe, W. P., *Science*, **142**, 483 (1963).
[14] Rowe, W. P., Black, P. H., and Levey, R. H., *Proc. Soc. Exp. Biol. and Med.*, **114**, 248 (1963).