EFFECTS OF HYPERVITAMINOSIS A ON FOETAL MOUSE BONES CULTIVATED IN VITRO

PRELIMINARY COMMUNICATION

BY

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It was shown in 1938 that vitamin A has the remarkable property of controlling the shape and texture of certain bones in young animals, so that when it is deficient or absent from the body these bones grow abnormally (Mellanby, 1938). The effect of this loss of ability of vitamin-A-deficient animals to control the shape and texture of bone is that the growing bones fail to adapt themselves to the developing nervous tissue and often destroy the nerves, both peripheral and central, by pressure. It was later shown that this dysplasia of bone is due to the fact that vitamin A has the function of controlling the position and intensity of activity of osteoclasts and osteoblasts (Mellanby, 1944). In some places normal osteoclastic and osteoblastic activity in growing bone of vitamin-A-deficient animals was reversed, so that where bone ought to have been removed it was laid down and vice versa, with disastrous effects on the nervous system. The restoration of vitamin A to the defective diets brought back the osteoclastic and osteoblastic activity to the normal position and the bone to a nearer normal shape.

The question was asked in the 1943 Croonian Lecture at the Royal Society whether this action of vitamin A was direct on the bones, or indirect by an action on some intermediary organ or humoral mechanism, and it was pointed out that an answer to this question could probably be obtained by tissue-culture experiments. This was one of the reasons why the present investigation was undertaken.

Although, in view of the foregoing results, it might be thought more natural to investigate the effect on bone growth of reducing the vitamin-A concentration in the culture media, for technical reasons it was found easier at first to test the effect of abnormally large quantities of this vitamin. As these experiments not only gave interesting results but also answered the question of whether the action of vitamin A on bone is direct or indirect, this preliminary account is being published.

The effect on bones of giving animals diets containing excessive amounts of vitamin A has often been observed. Collazo and Rodriguez (1933) and Bomskov and Seemann (1933) described the fragility and subsequent fracture of leg bones in rats given large amounts of vitamin A concentrates in their food. Similar observations were made by Davies and Moore (1934), Strauss (1934), and many others. It was later demonstrated that bone fragility and fracture could be produced by large doses of pure vitamin A (Herbst, Pavcek, and Elvehjem, 1944 ; Pavcek, Herbst, and Elvehjem, 1945 ; Moore and Wang, 1945).

As for the pathological processes in bone associated with the production of fragility and fracture, Strauss (1934) found that in hypervitaminosis A there was retardation of osteogenesis, reduction of endochondral bone formation, and a variable degree of osteoclasis only in the region of fractures. A more detailed description of the histology of this condition was given by Wolbach and Bessey (1942), who pointed out that the decalcification and osteoporosis were accompanied by a large number of osteoclasts, and that there was a greater degree of osteoporosis where the remodelling of bone was a normal process—namely, at the ends of long bones. Wolbach (1947), in studying the effect of hypervitaminosis A on bone in growing dogs, found that it accelerated the growth sequences. It caused rapid consumption of epiphysial cartilage and, in appropriate species, greatly premature closure of epiphyses and excessive rapidity of remodelling processes unrelated to linear growth of bone. He also found that the substitution of compact bone for cancellous bone in conformity to normal growth patterns was greatly accelerated, and stated that the fractures and sites of occurrence were fully explained by acceleration of remodelling.

Barnicot (1948) studied the direct action of vitamin A on bone by applying crystalline vitamin-A acetate locally to the bone. He found that it caused intense absorption by osteoclastic action, and suggested that the fractures observed in rats with hypervitaminosis A might be due to local action on the bones.

It will be seen in the following work that, in tissue cultures, bones under the influence of excessive vitamin A develop many of the changes described in the feeding experiments referred to above, and the results show that these changes are due to the vitamin acting directly on the bone.

Material and Methods

Material.—The tibiae, fibulae, radii, and ulnae of 17- to 21-day foetal mice were used. At this stage the epiphyses and the distal part of the diaphysis are cartilaginous, but for most of its length the shaft consists of a well-developed tube of bone enclosing a marrow cavity. These foetal mouse bones were particularly suitable for our purpose,

because their extremely small size made it possible to cultivate them *in vitro* at an advanced stage of development.

Tissue Culture.—The bones were dissected from the limbs, cleaned of all but a few tags of muscle, and grown by the watch-glass method described by Fell and Robison (1929). The culture medium consisted of a mixture of 6 drops of fowl plasma and 2 drops of a concentrated extract of 12- to 14-day chick embryo ; the embryo extract contained about 130 i.u. of vitamin A per 100 ml., so that the concentration of vitamin A in the plasma was thus diluted by from one-fourth to one-sixth in the final medium. The extract was made with Tyrode's solution to which 4% glucose and, to improve the calcification, 0.2% calcium glycerophosphate were added ; the addition of the glycerophosphate made it necessary to omit the acid phosphate to avoid precipitation, but the *p*H of the solution remained constant at 7.6. The explants were transferred to fresh medium every two to three days. Fresh embryo extract was made for each subculture and fresh plasma was usually drawn once a week.

For most of the experiments on the effects of " artificial " hypervitaminosis pure vitamin-A acetate in an alcoholic solution was added to normal plasma. In one experiment the physiological activity of vitamin A from " avoleum " (a commercial preparation kindly supplied by British Drug Houses, Ltd.) was compared with that of vitamin-A acetate. In order to study the effects of " natural " hypervitaminosis A, the explants were grown in plasma from fowls suffering from hypervitaminosis produced by a diet very rich in vitamin A.

Methods Used in Collection and Treatment of Bird Plasma.—For tissue-culture work it was necessary to obtain the blood and subsequently the plasma not only sterile but in such a condition that it did not clot until this was required—that is, until the embryo extract was added to
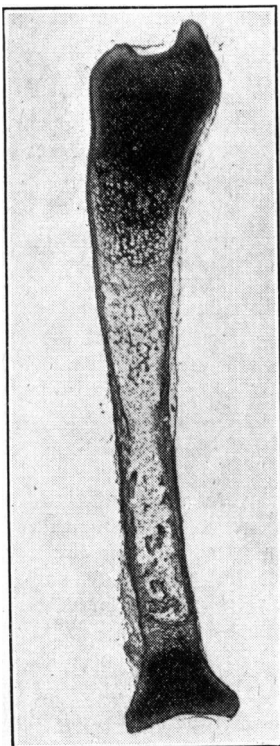

FIG. 1.—Radius from a late mouse foetus, cultivated in normal medium for seven days. The terminal cartilage is strongly basophil. (× 25.)

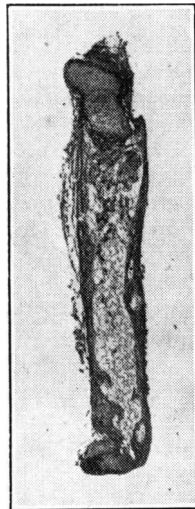FIG. 2.—R a d i u s from the opposite side of the same foetus as in Fig. 1, after seven days' c u l t i v a t i o n in medium containing 1,287–1,490 i.u. of vitamin A per 100 ml. of plasma. The cartilage has nearly disappeared and has completely lost its basophilia ; the bone a l s o i s b e i n g absorbed. (× 25.)

FIG. 3.—Tibia cultivated in normal medium for seven days. (× 25.)

FIG. 4.—T i b i a from the opposite side of the same foetus as in Fig. 3, after seven days' c u l t i v a t i o n in medium containing about 4,000 i.u. of vitamin A per 100 ml. of plasma. Very little cartilage or bone remains. (× 25.)

make the final culture medium. All the syringes and vessels used were sterilized and coated with peanut oil or paraffin wax. Precautions were also taken to chill the vessels, including the centrifuge tubes, and the blood and plasma at all stages. About 30 ml. of blood was drawn from the wing vein of each bird into a syringe containing 3 ml. of a heparin solution (10 i.u. per ml.), so that the blood contained about 1 i.u. of heparin per ml. It was immediately centrifuged and about 20 ml. of plasma was obtained from 30 ml. of blood.

Natural Hypervitaminosis-A Plasma.—Cocks were fed on diets very rich in vitamin A. For this purpose avoleum containing 30,000 i.u. of vitamin A per ml. was used : 5 ml. was given each day—2 ml. in the food and 1 ml. by mouth at 9 a.m., 2 p.m., and 5 p.m. Thus each bird received 150,000 i.u. of vitamin A daily. The normal plasma of control untreated birds contained about 300 i.u. of vitamin A and about 300 i.u. of carotene per 100 ml. In the birds fed for about 14 days, as described above, the vitamin-A content rose to 1,400–1,500 i.u. per 100 ml., while the carotene content diminished to about 125 i.u. per 100 ml. Whether the decrease was due to a diminished intake of carotene during the feeding period or to a direct action of the large amount, of vitamin A given is not known.

" Artificial " Hypervitaminosis-A Plasma.—This was obtained by adding an alcoholic solution of pure vitamin-A acetate of known strength to the normal bird's plasma. The amount added varied according to the degree of vitamin-A concentration in the plasma required, but the alcohol in the plasma never exceeded 1% and usually it was 0.5%. The same amount of pure alcohol was added to the normal control plasma used in the same experiment. In the earlier experiments vitamin-A acetate in alcohol was added to normal plasma to bring its content up to 4,000 i.u. or more per 100 ml. In all the later experiments, however, the content of vitamin A in this artificially treated plasma
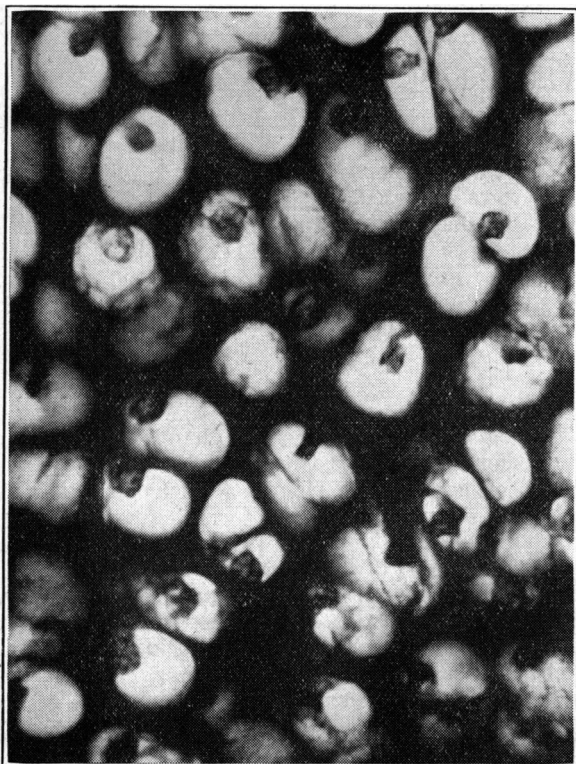
FIG. 5.—Area of terminal cartilage from the control tibia shown in Fig. 3. Note the broad, deep basophil partitions of matrix between the cells. (× 850.)

was only about 1,400–1,500 i.u. per 100 ml. to make it comparable with the natural hypervitaminosis plasma. In one experiment the vitamin A added to the plasma was obtained from avoleum : 8 ml. of alcohol was shaken up with 2 ml. of avoleum for about 5 minutes, centrifuged, and the clear alcohol, which contained about 6,000 i.u. of vitamin per ml., was withdrawn. A small amount of this solution was added to the normal plasma so as to bring the vitamin-A concentration up to the required amount. Here, again, the same amount of alcohol was added to the control plasma without added vitamin A.

Estimation of Vitamin A and Carotene in Bird Plasma.— These substances were determined in all the plasma specimens even where known amounts of vitamin A had been added to normal plasma. In such cases the calculated and determined results agreed fairly well. The method of estimation used closely followed that described by Yudkin (1941), a modification of Kimble's method (1939), and in detail was similar to that used by the Cambridge team in the investigation of the Medical Research Council (1949) on the vitamin-A requirements of human adults. Two modifications of detail were made : (*a*) a sealed glass tube was used instead of a corked centrifuge tube when the plasma and alcohol mixture was extracted with petroleum ether ; and (*b*) acetic anhydride to prevent turbidity in the absorptiometer was not added, as, under the experimental conditions employed, it was not necessary. The standard curves of reference used in the determination of carotene and vitamin A respectively were made from specimens of carotene and vitamin A which had been standardized in terms of international units.

Histological Technique.—Most of the explants were fixed in acetic Zenker's fluid and were serially sectioned. Some were fixed in absolute alcohol and mounted whole in glycerin jelly, a method which gave a very clear general picture of the bones.

Effect of Vitamin-A Acetate

In these experiments one of each pair of bones was explanted in medium containing the alcoholic solution of vitamin-A acetate, and the corresponding bone in normal medium containing the same amount of alcohol. The vitamin was added to produce concentrations ranging from 960 to 8,000 i.u. per 100 ml. of plasma. The vitamin-A content of normal bird plasma is about 300 i.u. per 100 ml. Forty-six pairs of bones were cultivated in this way.

The control bones in normal medium did not lengthen much during cultivation, but deposition and absorption of bone continued *in vitro*. During the first three days most of the haemopoietic tissue of the marrow degenerated, but usually the necrotic remains had almost disappeared by the fifth day, and the other tissues remained healthy for the longest period of cultivation (12 days). The soft tissue investing the bone grew profusely and formed a large zone of migrating cells.

The effect of the vitamin-A acetate on the experimental bones was remarkable. In a concentration of 2,000–4,000 i.u. per 100 ml. the soft tissue grew as richly as in the controls, but in three days the bone of the shaft began to be absorbed and the cartilaginous ends to soften and shrink. The process progressed rapidly, and the entire bone rudiment seemed to melt away like a lump of sugar in hot water ; in extreme cases, by the tenth day only a few crumbs of bone were left scattered in the sheet of actively growing soft tissue. It was noted that, the more healthy the culture appeared and the more actively it grew, the more dramatic was the effect of the vitamin-A acetate. It was rather toxic at a concentration of 8,000 i.u. per 100 ml., and produced a less striking result than was obtained at 3,000–4,000 i.u. per 100 ml.
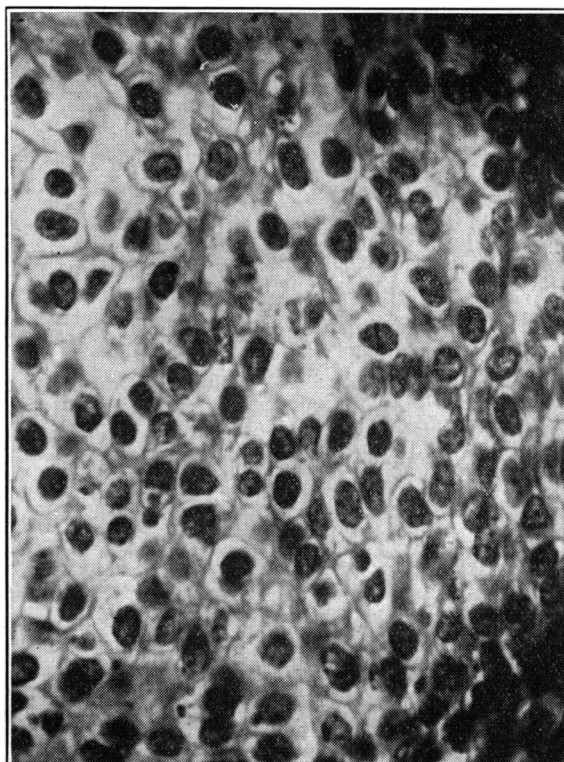

FIG. 6.—Area of terminal cartilage from the experimental tibia shown in Fig. 4. Note the healthy appearance of the cartilage cells, which are much closer together than in Fig. 5, owing to the almost complete disappearance of the cartilage matrix. (× 850.)

Histological sections of the experimental bones (Figs. 1–6), all stained with Delafield's haematoxylin and chromotrope, showed that the cartilage matrix soon lost its basophilia and at the same time shrank, while the cells appeared normal (cf. Figs. 5 and 6). Finally, the matrix of the epiphyses vanished completely, leaving only a compact mass of chondroblasts, while the diaphysial cartilage was rapidly invaded and replaced by the highly cellular connective tissue of the marrow cavity. Exactly how the bone was absorbed was not clear, and although osteoclasts were fairly numerous it was doubtful whether they accounted for all the destruction.

Effect of Vitamin A Prepared from Avoleum

One experiment was made in which "artificial" hypervitaminosis was produced by adding vitamin A in the form of an alcoholic extract of avoleum to the plasma. Six bones were grown in medium containing vitamin A from avoleum (1,558 i.u. per 100 ml. of plasma), six in medium containing vitamin-A acetate (1,538 i.u. per 100 ml. of plasma), and six in normal medium (286 i.u. per 100 ml. of plasma). For purposes of comparison, one bone of each pair was explanted in one of the experimental media and its fellow was grown in either normal or the other experimental medium.

The effect of the avoleum vitamin A seemed identical with that of vitamin-A acetate. By the fifth day the cartilage in both series of explants had begun to shrink and the bone to be absorbed, while the soft tissue grew profusely ; after nine days' cultivation the explants were only one-third to one-half the size of the controls, but the soft tissue was still growing actively.

Effect of Plasma from Fowls Suffering from Hypervitaminosis A .

Two experiments were made to find how the effect of pure vitamin-A acetate compared with that of the same concentration of vitamin occurring " naturally " in the plasma of birds suffering from hypervitaminosis A. Fowls were maintained on a diet containing large quantities of avoleum. When the vitamin A in the blood had reached a level of 1,200–1,500 i.u. per 100 ml. the plasma was drawn, mixed with embryo extract, and used as a culture medium.

In one experiment eight pairs of bones were explanted. The experimental member of each pair was grown for 12 days in plasma containing 1,435 i.u. of vitamin A per 100 ml. The control bones were cultivated in plasma from normal birds containing 487 i.u. per 100 ml. In the explants grown in the plasma from the birds with hypervitaminosis A the diaphysial bone appeared less dense than in the controls, but the difference was slight. This contrasted strongly with the results obtained in the experiments with pure vitamin-A acetate, in which a concentration in the plasma of only 950 i.u. per 100 ml. produced severe shrinkage of the cartilage and rarefaction of the bone after four to five days' cultivation.

In the second experiment the mouse foetuses were slightly younger than those in the first. Six bones were grown in plasma from fowls with hypervitaminosis A ; the plasma used for the first seven days of cultivation contained 1,235 i.u. of vitamin A per 100 ml. and that for the next five days 1,545 i.u. per 100 ml., a rather higher level than was reached in the first experiment. Six bones were grown in normal plasma to which approximately the same concentration of vitamin-A acetate had been added, and six were cultivated in normal medium (320 and 296 i.u. per 100 ml.). All three sets of plasma contained the same amount of alcohol. The experiment was arranged so that

one of each pair of bones was explanted in an experimental plasma, and one in either normal plasma or the other experimental plasma.

Apart from a slight rarefaction of the bony shaft, the explants grown in the plasma from birds with hypervitaminosis A showed little change for the first seven days ; then the cartilage began slowly to shrink and the bone to be extensively absorbed. The effect, however, was far less drastic than in the corresponding bones cultivated in the same concentration of vitamin-A acetate ; after 12 days the changes produced by the " natural " hypervitaminosis were about equivalent to those seen after four to five days' growth in the medium containing the same quantity of the acetate.

Discussion

These results showed that when vitamin A in tissue culture medium is increased from about 300 to 1,200 i.u. per 100 ml. by adding pure vitamin-A acetate to the plasma there is a dramatic effect on foetal bones explanted in such a medium. Plasma drawn from birds with natural hypervitaminosis A of the same order—that is, 1,200–1,500 i.u. per ml.—produced by a diet containing an excess of this vitamin, affects bone explants in a similar way, but its action is much less drastic than that of the artificially enriched plasma. What this quantitative difference in action is due to is not known.

One of the most striking observations described above is the disintegrating effect on the cartilage of foetal mouse bones of vitamin A in abnormally high concentrations. This action is primarily on the matrix, which softens, shrinks, and loses its basophilia, while the cartilage cells remain normal in appearance. In the hypertrophic cartilage at the ends of the diaphysis these changes are associated with a very rapid invasion and replacement of the cartilage by the tissue of the marrow cavity.

This destruction of cartilage in vitro is clearly related to the changes produced in cartilage in vivo by hypervitaminosis A and described by Strauss (1934), Wolbach and Bessey (1942), and Wolbach (1947). In both conditions there is abnormally rapid excavation of hypertrophic cartilage by the tissues of the marrow, but in the present work the most conspicuous effect is the dissolution of the matrix in the uninvaded cartilage, while in Wolbach's description the chief feature is an acceleration of the normal cartilaginous sequences. Again, in both the in vivo and in vitro experiments there is destruction of bone, but in the explants nearly all the bone eventually disappears, probably only partly by osteoclasis, whereas Wolbach states that in vivo the destruction is limited to osteoclasis in those regions where shaping of the bone normally takes place. Thus, although the effects of hypervitaminosis A are of the same general character in vivo and in vitro, there are considerable differences in detail. This is probably due to the very different environmental conditions in vitro and in vivo, and especially to the absence of a blood circulation in the explanted bones.

Summary

Long bones from late mouse foetuses were cultivated by the watch-glass method in medium containing abnormally high concentrations of vitamin A.

When pure vitamin-A acetate was added to the culture medium in concentrations of 1,000–3,000 i.u. per 100 ml. the matrix of the terminal cartilage softened, shrank, and finally almost or completely disappeared, though the cartilage cells appeared normal ; in the shaft the cartilage was rapidly replaced by marrow tissue ; the bone was resorbed, sometimes

completely ; the soft tissue surrounding the explant grew as vigorously as in the controls cultivated in normal medium.

The same results were produced by similar concentrations of an alcoholic extract of avoleum, a commercial preparation rich in vitamin A, made from liver.

Similar concentrations of vitamin A obtained by using plasma from fowls fed on a diet rich in the vitamin had a much less drastic effect on the explants.

We are indebted to Mr. L. J. King, technician at the Strangeways Research Laboratory, for his assistance with the tissue culture; to Mr. R. J. C. Stewart, chief technician of the Nutrition Building, National Institute for Medical Research, for his valuable help with the preparation of the birds' plasma; and to Mr. V. C. Norfield for the photographs.

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Speaking at the fourth annual conference dinner of the Society of Radiographers, Sir Andrew Davidson, Chief Medical Officer, Department of Health for Scotland, said that the Health Service Acts had made profound changes in the administration of the health services—changes made necessary mainly because of new and progressive trends in medical science and a fuller appreciation of the social, economic, and industrial effects of sickness and disability. There were many gaps to be filled and many deficiencies to be repaired. The new health service claimed to be doing that. Many agreed that the changes had brought real and appreciable public benefits ; but they heard less of them than of the weaknesses. The new arrangements had immensely widened the opportunities for the prevention and treatment of ill-health wherever it arose. But could they take advantage of the opportunities ? It was of great advantage that Scotland was not too large to prevent personal contacts, yet large enough to be able to maintain a full range of service. Important also was the happy circumstance that they had four flourishing medical university centres, all of the first rank, which together produced about one-third of the new doctors in the United Kingdom. It was fortunate also that the pattern for medical care in rural districts had been set up by the Highlands and Islands Medical Service. Again, Scotland had a well-developed public health service in the counties and burghs. It was too early to make any general assessment of positive achievements, but two facts stood out: (1) The Service has been accepted and indeed welcomed by the public, as 97% of the population are included on doctors' lists ; and (2) those who were concerned in operating the Service were making strenuous and helpful efforts to make it a success. The proper balance between the various elements of the Service must be struck. More attention would need to be paid to those working in the homes of the people, whether they were concerned with prevention or cure. The co-operation of the public was needed on how to use the Service. Abuses would destroy it. Doctors had told him that much of the expenditure on expensive drugs was in response to demands by many patients for what they themselves wanted. Surely that was wrong. The public should not regard the Service merely as a machine which would turn out medicine, or spectacles, or dentures, or any other apparatus on demand.

RESPIRATORY STUDIES IN PARALYTIC POLIOMYELITIS

BY

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The following notes are based on studies at the Western Hospital, Fulham, by two of us (L. M. T. and E. D.-J.) during the 1949 epidemic of poliomyelitis, and on laboratory studies at Stoke Mandeville Hospital. While many of the conclusions are tentative, it has become clear that there is much need for improvement in both the design and use of breathing machines. These notes are published in the hope of facilitating such improvement.

The tidal air of a patient in an iron lung can be measured without difficulty by the use of a spirometer of the type commonly employed in hospitals for the measurement of basal metabolism. There are, however, few published measurements of this type in cases of poliomyelitis, and very few detailed measurements taken from patients in tank respirators. Drinker and Shaw (1929) and Shaw and Drinker (1929) showed that in healthy subjects in tank respirators it was easy to produce a large tidal air with minute volumes of over 20 litres a minute at pressures of 20 cm. of water. Elam (1948) showed figures for six cases of poliomyelitis in respirators, but gave few details of the patients' condition. Binet, Bochet, and Bour (1948) gave figures for one patient and four healthy subjects.

Our studies have been concerned chiefly with the effects on tidal air and minute volumes of varying respirator suction pressures and stroke frequency. We have also studied the effects of varying the patient's posture while in a respirator, and the changes occurring in convalescent patients during and after weaning from their respirators. These experiences have indicated a need for a number of alterations in the Both type of respirator, which are discussed later.

Technique

1. *Spirometers.*—A commercial spirometer, using a rubber bellows instead of a float, was found to give too high a resistance to air flow for accurate work. A spirometer of the water-sealed gasometer type was therefore constructed. The counterweight guides and pulley were fitted with ball-races to reduce friction, and thus the pressure required to start the spirometer moving was reduced to only 2 mm. H_2O. As the spirometer was required to measure much smaller tidal airs than normal, the counterweight pulley system was designed to give an optional multiplication ($\times 2$) of the float movement. The sensitivity when this was used was 3.67 in. (9.32 cm.) per litre. The spirometer included an absorbent for CO_2, and was refilled with pure oxygen as and when necessary.

2. *Recording Drum.*—The recording drum used with this spirometer was 12 in. (30 cm.) in diameter and 16 in. (40 cm.) high, and gave paper speeds variable at will from about 2 in. (5 cm.) a minute to 1 in. (2.5 cm.) a second. The highest speed was adequate to display clearly the wave-form of each respiration.