fat composition changes in the 0° group.

Discussion. These results indicate that body fat composition can be altered upon changing environmental temperature even in a species that is well insulated externally, and in absence of a correlation with body temperature. The body temperature gradient noted in the pig may therefore be only incidental to the changes observed in fat composition, while altered metabolic activity resulting from acclimation to cold and warm environments may more likely be responsible.

We have previously shown(5) that degree of unsaturation of body and liver fat increased on starvation as a result of the preferential retention of polyunsaturated fatty acids. At 0°C temperature the hens in this study lost body weight (8.5%) during adjustment to their new environment. However, this weight loss, which resembles the loss during starvation, does not explain completely the greater unsaturation of the depot fat from the birds maintained in the cold, because the hens kept at 32°C also lost weight (10.8%) during the adjustment period without concomitant change in body fat composition. The changes in body fat composition are also probably not related to differences in reproductive activity, for while the birds at 0°C were below controls in rate of egg production (56% *vs* 78%), the birds at 32°C also produced at a reduced rate (62%).

While the exact mechanism of the observed change is not clear, it is of interest to point out that Henriques and Hansen(1) believed (without citing data) that the greater unsaturation in pig depot fat was due to an increase in oleic acid concentration. In the present study oleic acid actually decreased while the dienoic and hexaenoic acid fractions increased.

Summary. Samples of subcutaneous body fat were analyzed for fatty acids and iodine values in hens maintained at 3 environmental temperatures. It was found that fat from hens maintained at 0°C was significantly more unsaturated and contained more di- and hexaenoic acid than fat from birds kept at either 21° or 32°C. There were no changes in deep body temperature or temperature of the tissue at site of fat biopsy which correlated with the changes in fat composition. It is suggested that metabolic adjustments associated with cold adaptation are responsible for the changes in body fat composition.

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1. Henriques, V., Hansen, C. C., *Skand. Arch. Physiol.,* 1901, v11, 151.

2. Hilditch, T. P., *The Chemical Constitution of Natural Fats,* John Wiley & Sons, New York, 1956, p456.

3. Assn. of Official Agricultural Chemists, 1955, *Official Methods of Analysis,* 8th ed., Washington, D. C.

4. Luddy, F. E., Barford, R. A., Riemenschneider, R. W., Evans, J. D., *J. Biol. Chem.,* 1958, v232, 843.

5. Feigenbaum, A. S., Fisher, H., *Brit. J. Nutr.,* 1962, in press.

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Application of Direct and Indirect Immunofluorescence for Identification of Enteroviruses and Titrating their Antibodies.* (27664)

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The reports dealing with immunofluorescence of enteroviruses have dealt with either the time sequence of events in tissue culture cells after infection with the agents or with identification of unknown enteroviruses. Buckley(1), using the indirect procedure, stained the 3 types of poliovirus in tissue culture and noted the time and morphology of newly formed virus antigen within tissue culture cells. Other workers(2,3,4) using different tissue culture systems and modifica-

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tions of the fluorescent antibody (FA) procedure (direct, indirect, and complement staining) have correlated the infectious process with formation of viral antigen and newly formed infectious virus, and have also studied the different antigens of a single type of virus. Hatch *et al.*(5) used the direct staining procedure to identify virus isolates from stool specimens and found good correlation between the FA procedure and conventional methods used in identification of unknown viruses. The procedure has also been used to identify other enteroviruses isolated from clinical specimens(6,7,8).

In view of the potential usefulness of fluorescent antibody technics with all the enteroviruses, a study of both the direct method for identification of enteroviruses and the indirect method for determination and titration of serum antibodies against these agents is presented here.

Direct method. Specific hyperimmune rabbit or monkey sera were first subjected to crude fractionation with half saturated ammonium sulphate and were then conjugated with either fluorescein isothiocyanate(9) or with a new dye, tetramethyl rhodamine isothiocyanate.† After dialysis to pH 6.3 in 0.0175 M PO_4 buffer, the conjugate was further purified by fractionation on a diethylaminoethyl cellulose (DEAE) column which was 2 cm in diameter and 20 cm in height. This latter procedure eliminates any remaining traces of albumin, as well as most alpha and beta globulins, and retains only the active fraction of the gamma globulin. HeLa or monkey kidney cells infected with stool isolates, or with laboratory strains of poliomyelitis, Coxsackie or ECHO viruses, were either scraped off the glass or, preferably, grown as coverslip cultures. The cells were fixed with acetone and stained directly with labeled antisera for one hour at 37°C. After washing with phosphate buffered saline at pH 7.2, the preparations were mounted in buffered glycerol and examined under a Zeiss binocular microscope with a dark field condenser using an Osram HBO 200 light source. The filter system consisted of a UG-5 exciter filter and either a GG-4 barrier filter alone or in combination with an OG-4 barrier filter. Fig. 1 illustrates several examples of type specific reactions as compared with appropriate controls. Autofluorescence from tissue artifacts, uninfected cells, or cells infected with heterologous types of virus was sometimes seen but the specific reactions appeared definitely brighter and more distinct.

Indirect method. The indirect method of immunofluorescence has certain advantages over the direct method in that nonspecific reactions are generally less prominent with increasing dilutions of the intermediate serum and in that only one final labeled antiserum is needed to test for various types of antibodies (Fig. 1). Furthermore, this procedure enables one to detect type specific antibodies in small quantities of serum and to measure the antibodies by progressive dilutions of the specimen. In our laboratory a convenient application of this technic consisted of adding one or 2 drops of varying dilutions of serum to coverslip cultures of infected cells and incubating the slides overnight at 4°C. The next morning after removing the serum and washing with phosphate buffered saline at pH 7.2 the slides were stained for one hour at 37°C with final labeled antiserum prepared against the species of the intermediate (unknown) serum. Upon microscopic examination reactions were graded 4+, 3+, 2+, 1+, or negative depending upon the brilliance of the fluorescence. FA titers were defined as the highest dilution of the intermediate serum with which the specific conjugated antiserum gave a 4+ reaction. Very low dilutions of human sera with known neutralizing antibody titers to one or more viruses appeared to give nonspecific cross or group reactions with other agents. However, with dilutions of 1:8 or higher, type specific reactions were clearly distinguishable. There was a direct relationship between the neutralizing antibody titer and the extent to which the serum could be diluted and still react, but the fluorescent antibody (FA) titer was consistently lower. In fact, at least a 16-fold difference was usually observed. The representative results presented in Table I show a comparison of

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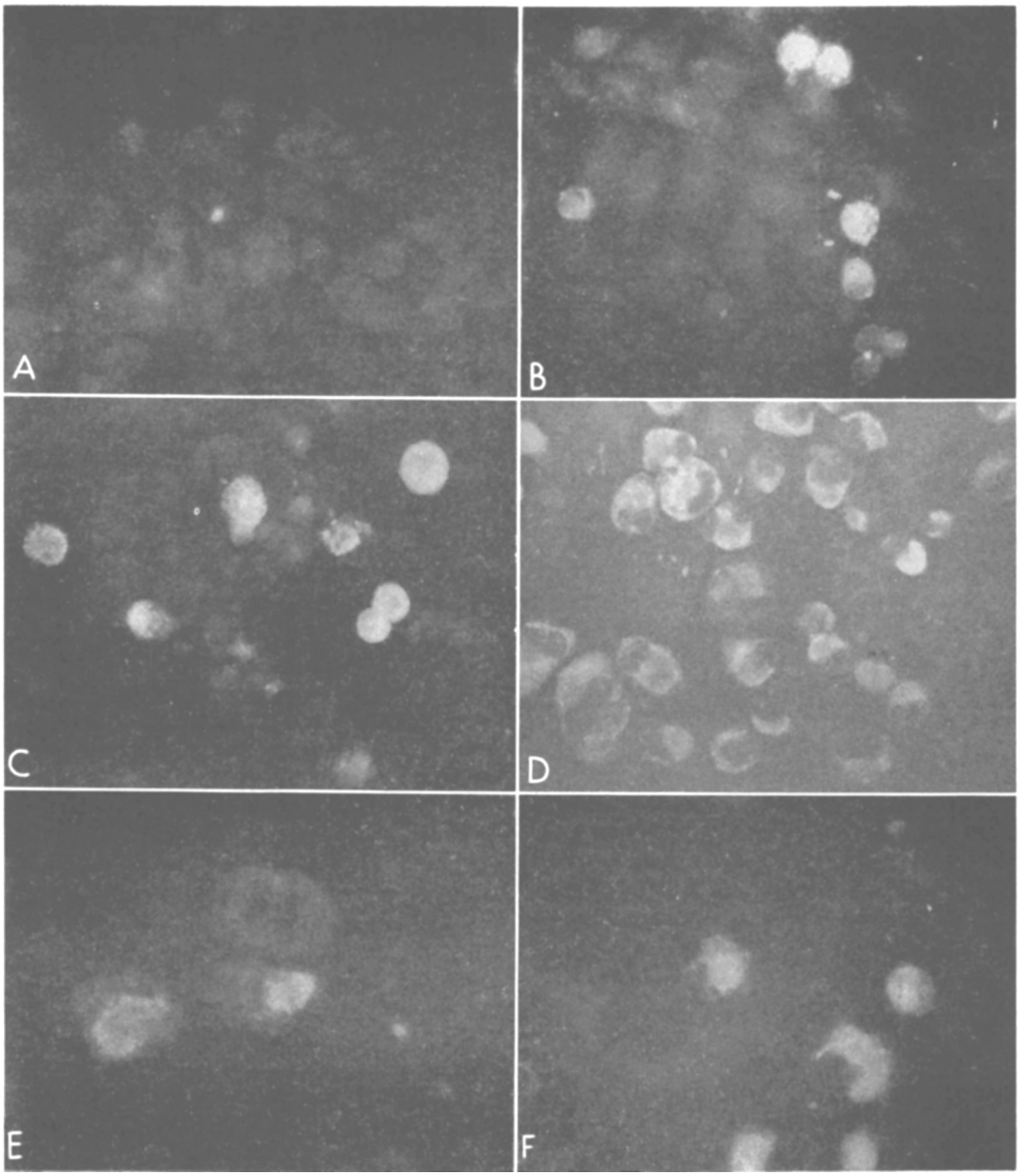

FIG. 1. A. Control—HeLa cells infected with poliovirus type II, stained by direct method with fluorescein labeled anti type III antibody (× 400). B. HeLa cells infected with poliovirus type I, stained by direct method with rhodamine labeled anti type I antibody (× 400). C. HeLa cells infected with poliovirus type II, stained by direct method with fluorescein labeled anti type II antibody (× 400). D. HeLa cells infected with poliovirus type III, stained by indirect technic. Intermediate (patient's) serum was diluted 1:32, and final serum was fluorescein labeled anti-human gamma globulin (× 400). E. HeLa cells infected with Coxsackie B-1 virus, stained by indirect technic. Intermediate (patient's) serum was diluted 1:16, and final serum was fluorescein labeled anti-human gamma globulin (× 400). F. Monkey kidney cells infected with ECHO-9 virus, stained by indirect technic. Intermediate (patient's) serum was diluted 1:32, and final serum was fluorescein labeled anti-human gamma globulin (× 400).

TABLE I. Comparison of Virus Neutralizing and Fluorescent Antibody (Indirect) Titers of Human Sera.

Virus	Neut.	FA
Polio I	64	16
	256	16
	2048	32
Polio II	64	8
	256	16
	1024	16
Polio III	256	16
	1024	32
	2048	64
Cox. B-1	256	32
B-2	64	16
	256	32
B-3	128	32
	64	16
B-4	128	32
	512	16
B-5	128	32
ECHO-9	256	64
	1024	32

the neutralization and indirect FA titers obtained with human sera containing antibodies acquired as a result of subclinical infections with various enteroviruses. The specificity of these reactions is illustrated in Fig. 2 which shows the result of testing 2 sera against 5 types of Coxsackie B virus. One specimen (P.H.) contained demonstrable neutralizing antibodies for only one type, Coxsackie B-3, and another (M.G.) for 2 agents, Coxsackie B-2 and 4. The homologous reactions are in each instance clearly defined by the higher

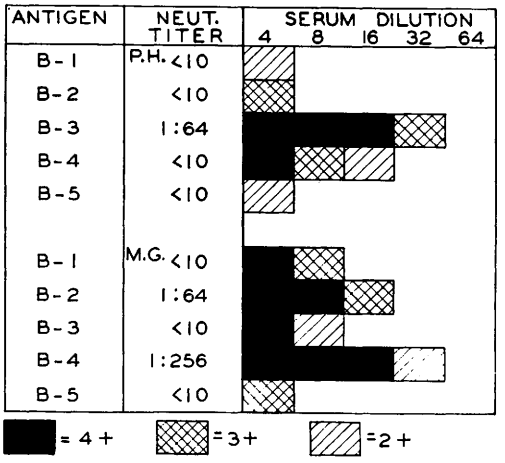

FIG. 2. Identification of Coxsackie antibodies in human serum by indirect immunofluorescence.

dilution of serum giving a 4+ stain. The importance of discounting results with dilutions of less than 1:8 is illustrated by the frequency of nonspecific reactions at the 1:4 dilution. The specificity of reaction is further illustrated in Fig. 3 which shows the FA titers of

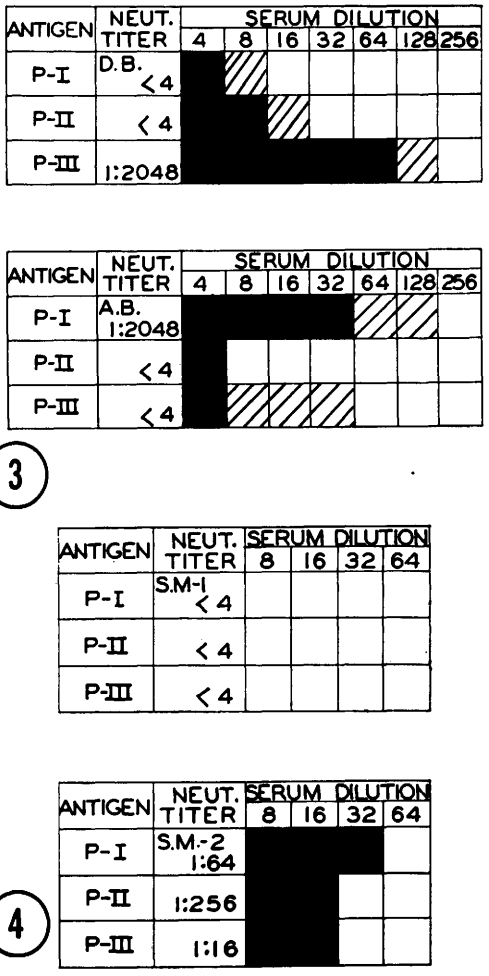

FIG. 3 and 4. Identification of polio antibodies in human serum by indirect fluorescence microscopy (labeled goat anti-human gamma globulin).

sera of persons convalescent from virus confirmed paralytic poliomyelitis infections. In like manner the active antibody response to inactivated poliomyelitis vaccine can be measured by comparing the FA titers of pre and post vaccine sera (Fig. 4). Coincident with these studies it was observed that sera of young infants containing maternally transferred, passive antibodies in high titer do *not*

react with the same labeled antiserum, either prior to or following the vaccination procedure. These results are being reported elsewhere(10).

Summary. Tissue culture cells infected with various enteroviruses show specific immunofluorescence when stained directly with conjugated fractionated antiserum. The indirect method of fluorescent antibody determination is an even more satisfactory diagnostic technic since it makes possible the detection and titration of enterovirus antibodies actively acquired following clinical or subclinical infection or from vaccination.

1. Buckley, S. M., *Am. J. Path.,* 1957, v33, 691.
2. Mayor, H. D., *Texas Rep. Biol. and Med.,* 1961, v19, 106.
3. Hinuma, Y., Hummeler, K., *J. Immunol.,* 1961, v87, 367.
4. Levy, H. B., *Virology,* 1961, v15, 173.
5. Hatch, M. H., Kalter, S. S., Ajello, G. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 1.
6. Hatch, M. H., *Bact. Proc.,* 1962, p137.
7. Page, R. H., Stulberg, C. S., *ibid.,* 1962, p137.
8. Shaw, E. D., Newton, A., Powell, A. W., Friday, C. J., *Virology,* 1961, v15, 208.
9. Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., Metcalf, T. G., *Am. J. Path.,* 1958, v34, 1081.
10. Riggs, J. L., Brown, G. C., *J. Immunol.,* in press.

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Relationship Between Soluble Collagen and Urinary Hydroxyproline in the Lathyritic Rat.* (27665)

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Ziff and coworkers(1) have demonstrated increased excretion of urinary hydroxyproline peptides in children as compared with adults. Decreased excretion has recently been observed in this laboratory in non-growing children (2). This was corrected in a pituitary dwarf by administration of human growth hormone. Increased levels of urinary hydroxyproline were observed in a patient with active acromegaly(2), and in animal experiments, elevated values were also found in young rats and guinea pigs as compared with adults(3,4). A relationship between rate of growth and size of the soluble collagen pool has also been demonstrated in animals(5-8). The latter finding, taken together with the evidence previously cited, suggests that the level of excretion of urinary hydroxyproline peptides may be related to the size of the metabolically active pool of soluble collagen as previously proposed(1).

A striking increase in the amount of extractable, neutral salt soluble collagen has been demonstrated in several animal species following administration of lathyritic agents (9-13). This has occurred without detectable increase in the rate of synthesis of total collagen(10,13). In the light of this increase, the excretion of hydroxyproline has been measured in lathyritic animals in an effort to gain additional evidence for a relationship between amount of urinary hydroxyproline peptides and size of the soluble collagen pool under circumstances in which growth was not the primary factor in enlarging this pool. In the following experiments, simultaneous determinations of the neutral salt soluble collagen of the skin and urinary hydroxyproline have been made in lathyritic and control rats. A recent preliminary report(4) has demonstrated the presence of increased amounts of hydroxyproline in the urine of animals with lathyrism.

Experimental. Twenty-eight Sprague-Dawley rats (Holtzman) from 3 litters, delivered within 72 hours of each other, were weaned at

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